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EXPERIMENTAL SYPHILIS IN THE RABBIT.

V. Syphilitic Affections of the Mucous Membranes and Mucocutaneous Borders.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 78 to 83.

(Received for publication, June 14, 1920.)

A study of cutaneous syphilis in the rabbit brought out the fact that although the infecting organisms might be widely distributed through the body, lesions of an easily recognizable character occurred almost exclusively within certain restricted areas while the remainder of the skin surface rarely showed any manifestation of disease (1, 2). In like manner, it has been found that lesions develop with considerable frequency in parts of the body where skin and mucous surfaces join. In some instances, the lesions first appear within the skin area, while in others they develop upon the mucous membrane so that it might be possible to classify most of them as affections of one or the other of these structures. It appears, however, that the transitional area exercises some influence upon the localization of the infection, and since lesions which develop in one tissue usually extend to the other, their classification as affections of mucocutaneous borders seems to be a more logical one.

Syphilitic involvement of the mucous membranes and mucocutaneous borders of the rabbit was first noted by Grouven (3), who described infiltrations of the nasal mucosa and rhagades about the nasal orifices, conjunctivitis, and papular lesions of the anus and sheath. Attention was also called to the presence of a nasal discharge containing spirochetes and to dyspnea presumably resulting from infiltration and swelling of the nasal mucosa. These early observations have been confirmed and amplified to some extent by subsequent observers, but no material additions have been made to the list of conditions originally described by Grouven. Uhlenhuth and Mulzer (4) described tumor-like swellings of the mucous membranes following generalized inoculations, and within recent

years, attention has been directed more particularly to the occurrence of diffuse inflammatory processes of the nose and sheath which show no characteristic lesions but are identified by a mucopurulent discharge which contains spirochetes. These observations constitute the chief contributions which have been made to the study of affections of the mucous membranes and mucocutaneous borders in the rabbit.

Among the animals first studied by us, localized infections of the mucous membranes and mucocutaneous borders were noted in about 20 per cent of the cases but have been less frequent among those studied more recently. The affections seen in these animals were of two general classes, depending upon the type of the lesions present. In one group, the lesions were characterized by diffuse infiltration, surface erosion or ulceration, and the formation of exudates of various types; in the other, there was a greater degree of proliferation and the lesions formed were large granulomatous masses which showed the usual secondary transformations of syphilitic processes. Affections of these two classes were distributed about the nares, the lips, the margins of the lids, the genitalia, and the anus.

Affections of the Nasolacrimal System and of the Nasolabial Region.

Infections of the nasolabial region were relatively infrequent, which, as will be explained later, may have been due to the fact that lesions in these areas were of comparatively late development and the animals were not held for a sufficiently long time.

The most common affection seen was a condition which in its earlier stages closely resembled an ordinary case of snuffles. The infection began as a rhinitis with a more or less profuse mucopurulent discharge and apparently extended to the nasolacrimal ducts, producing obstruction and consequent overflow of the lacrimal secretion.

At the onset of the local infection, no characteristic lesions could be detected by ordinary means of examination, but within a short time the discharge from the nose became of a more tenacious character and tended to adhere to the surfaces about the nasal orifices. There was then a noticeable reddening and swelling of the skin and mucous membranes, and removal of the accumulated discharge revealed the presence of minute abrasions as indicated in Fig. 1. An earlier stage of the process may also be seen in Fig. 9 which is a photograph of the same animal as that in Fig. 1, taken 1 month earlier.

As the local infection advanced, the skin areas became denuded of hair; the infiltration of the surrounding tissues was increased, especially along the edges of the alæ nasi, and the nares were obstructed by the presence of accumulated discharges or by thick adherent crusts, the removal of which left numerous raw and bleeding points (Figs. 1 and 2).

Several modifications of this general type of condition were seen. In some animals, the nasal discharge was comparatively slight, and the lesions were of a less diffuse character. Irregular areas of infiltration and rhagades occurred along the margins of the alæ and more especially at the angles of the nares. Occasionally also, simple areas of ulceration or ulcers surrounded by a definite zone of infiltration such as that in Fig. 3 were seen just at the edges of the nasal orifices.

Another very characteristic affection of the nasolabial region is that shown in Fig. 4. The condition as presented by this animal was a marked infiltration of the nasal mucosa and the tissues surrounding the left nasal orifice. The right side of the nose was also affected to a slight extent. The cutaneous portion of the lesion was covered by an accumulation of epithelial scales and crusts, while the mucous surface showed ulcers with a gray necrotic base.

An unusually destructive condition seen in one animal of this group is that shown in Figs. 5 to 8. The usual symptoms of acute rhinitis were absent in this case. When first detected a circumscribed, indurated mass was present in the superior portion of the right nostril and a smaller area in the left (Fig. 5). These lesions increased very rapidly, giving great breadth and prominence to the alæ (Figs. 5, 6, and 10). Depressed ulcers developed upon the mucous surfaces and spread until the skin margins were involved. Heavy reddish brown crusts were then formed and practically occluded the anterior nares on both sides (Fig. 7). The necrosis associated with these lesions was quite marked and led to considerable destruction of the soft tissues and consequent deformity of the nose (Fig. 8). At autopsy, it was found that a large part of the mucous membrane of both nasal passages had become involved and the left chamber was practically obliterated for a distance of about 2 cm. above the anterior orifice. Whether this was due to the *pallidum* infection or was the result of an associated bacterial infection could not be determined.

One other form of lesion was observed at the margins of the nares, and this also was seen in but a single animal. The lesion in this case was a discrete papule about 3 or 4 mm. in diameter situated on the lip near the median line. The tumor-like swellings of the nasal mucosa described by Uhlenhuth and Mulzer (4) were not observed in any of these animals. They have been noted by other observers (5), however, and it is possible that some of the conditions which we have described as infiltrations of the nasal mucosa may represent processes analogous to those referred to as nasal tumors.

Taken as a whole, this group of conditions presented a very characteristic appearance and resembled in many respects the nasal

affections of infants with hereditary syphilis. The essential feature of the lesions was an infiltration of the skin and mucous membranes with irregularly distributed areas of necrosis and ulceration, while the characteristic symptoms were the presence of a mucopurulent discharge, epiphora, or conjunctivitis, and dyspnea, the latter being apparently an obstructive phenomenon.

Lacrimation or lacrimal overflow has been mentioned by different writers as a symptom of infection of the nasal mucosa, and while there was a very constant association of these conditions, the causative connection between the two processes was not entirely certain. During the early stage of the nasal infection, the characteristic condition of the lacrimal system was that shown in Figs. 9 and 10. At this period, the cheeks below the anterior angle of the eye were bathed with a clear lacrimal secretion. The hair was matted together, the skin was slightly inflamed, and usually there was some loss of hair over these parts. The conjunctiva in some instances remained perfectly clear, while in others there were varying degrees of an acute inflammatory reaction. Eventually, the lacrimal secretion was altered, becoming more clouded, or mucopurulent, in character. The conjunctivæ were then dulled, and the secretion accumulated over the cheeks in greater amount as in Fig. 11.

Other workers have reported the presence of spirochetes in the lacrimal secretions, but repeated examination in these cases failed to show them. While this in itself is not conclusive, it suggests that these affections might be referable to occlusion of the nasolacrimal ducts either as a result of involvement of the nasal mucosa or of infection of the ducts themselves rather than to localized infection of the conjunctival sac. Except for its periodicity and its persistence, this feature of the nasal infection differed in no way from a similar condition frequently observed in rabbits from other causes.

Occurrence and Duration.—As has been stated, affections of the nasal region were not among the early manifestations of generalized syphilis. The majority of those seen were either late in their development or at least occurred subsequently to other types of lesions. The time at which they were first recognized varied from a minimum of 9 weeks to a maximum of 8 months after inoculation, but there were very few cases in which lesions appeared within the first 4 months.

These statements may give an erroneous impression as to the time at which the infection actually became localized in the mucous membranes of the nose or about the mucocutaneous margins, since the early

symptoms were in most cases but little more than an ordinary rhinitis and specific infection was recognized only after the condition had advanced sufficiently to arouse suspicion as to its etiology. For the same reasons, many cases of nasal infection may have passed unrecognized, since it was not possible to make routine examinations of all nasal discharges.

This group of lesions was not only late in developing but was also of a very enduring character. With a few exceptions, the animals of this group were held for a number of months after the development of the localized infection, and no instance was recorded of complete healing of the lesions during the period of observation. Two of the animals were held for approximately 1 year, and a third was under observation for more than 2 years. In the last animal, there were several periods during which the lesions about the nares underwent almost complete resolution, but each time there was a recurrence. It seems probable, therefore, that this group of conditions may be regarded as among the most persistent of the generalized infections in the rabbit.

Affections of the Lips and Buccal Cavity.

Syphilitic lesions were occasionally noted upon the mucous surfaces or along the margins of the lips, but the entire buccal cavity of the rabbit is an almost unexplored region. Small papular infiltrations were seen on the skin surfaces or at the mucocutaneous borders of both the upper and lower lips as described in the fourth paper of this series (1, 2), but even these were rare. In addition, lesions were found about the cleft in the upper lip or upon the mucous surfaces of the lips.

The region of the cleft in the upper lip was one of especial interest. Normally, the contact surfaces of this area are covered by a short downy growth of hair which reaches practically to the inner margins as shown in Fig. 12. There were several animals in which an infiltration about the nasal orifices continued downward along the margins of the nasolabial folds, forming a thickened ridge, the surface of which was bare and covered by scales or by gray necrotic patches and small moist areas of erosion. A condition of this kind was present on the left lip of the animal shown in Fig. 4.

In addition to such processes as these, there were a few instances in which independent affections of the cleft were observed. The lesions appeared in the form of small papules or flattened patches of infiltration which tended to spread over the surface of the lips, and the resulting affection presented a very characteristic appearance which is shown in Fig. 13. It will be noted here that the affected portions of the lips are moist and denuded of hair, that there is a distinct thickening of the left lip, and that the surfaces are marked by an irregular network of ridges covered by gray necrotic areas. Eventually small superficial ulcers were formed which bore a striking resemblance to the mucous lesions of man.

Similar affections were also noted on the mucous surfaces of the lips, but, as a rule, these were small erosions of an indifferent character in which no spirochetes could be demonstrated. In a single instance, however, an ulcer with definitely infiltrated margins was found upon the inner surface of the upper lip (Fig. 14).

Another type of condition which was seen in a number of rabbits consisted of small papillomatous growths which were occasionally present on the margins or inner surfaces of the lips, about the gums of the lower incisors, and were especially numerous on the sides and under surface of the tongue. While these affections resembled in some respects certain of the hypertrophic or vegetating lesions of man, we could obtain no proof of a syphilitic origin—either clinical or histological.

Occurrence and Duration.—Affections of the lips were in part no more than extensions from those about the nose and were subject to the same general conditions. The independent affections of the lips appeared to be of earlier development and of comparatively short duration. There were very few of these, however, and too much cannot be inferred from such a small group of cases.

Affections of the Eyelids.

The lesions seen on the eyelids might be separated into two distinct classes according to their location, those which apparently originated in the skin and seemed to bear no particular relation to the marginal area, and those which originated at the mucocutaneous junction of the lid or ultimately involved this area. The first group of conditions was described in the paper dealing with cutaneous lesions. The affections of the mucocutaneous borders proper were also of two general types, first, papular or granulomatous lesions (Figs. 15 and 16), and second, lesions which appeared in the form of ulcers along the margins of the lids (Fig. 17).

The papular and granulomatous lesions presented no essential difference from the skin affections previously described, except as they came to involve the conjunctival surfaces. When this occurred, there was usually a moderate degree of conjunctivitis with reddening and swelling of the conjunctiva and increased lacrimation. These symptoms, however, were only transient, and within a few days, such inflammatory reactions were confined to the immediate area of the lesion (Fig. 16).

Lesions which originated upon the margins of the lids or on the surface of the conjunctiva itself usually produced a greater degree of inflammatory reaction. In most instances, they appeared, as we have said, in the form of small ulcers or abrasions. Occasionally, the initial affection was a minute papule, such as that in Fig. 15, which subsequently underwent ulceration. The appearance presented in these cases depended largely upon the extent of the induration which was associated with the formation of the ulcer. In several instances, lesions of this type developed definite collars of induration such as that shown in Fig. 17. As will be seen from this figure, there was a well marked area of infiltration extending from the margin of the lid back under the surface of the conjunctiva.

Occurrence and Duration.—In the few animals which showed lesions upon the margins of the lid, the time of appearance of these lesions varied from about 3 to 11 months after inoculation, but the majority occurred within 4 months. On the whole, they were decidedly less enduring than those about the nose, but in exceptional instances, they lasted for several months with varying periods of activity and quiescence or regression.

Affections of the Penis and Sheath.

As in the case of localized infections about the nose, it is probable that many cases of specific infection of the penis and sheath occurred which did not lead to the formation of lesions of a sufficiently definite character to attract our attention. Among the animals in this group, there were several in which the diagnosis of the local infection was made prior to the development of any characteristic lesion. These cases were suspected on account of redness and some swelling of the sheath and the presence of a mucopurulent discharge which upon examination was found to contain *Treponema pallidum*. Otherwise the diagnosis was first made on account of the presence of lesions which proved to be syphilitic.

The list of affections which occurred about the penis and sheath included first, cases of a diffuse inflammatory character associated with a mucopurulent exudate, second, circumscribed or diffuse infiltrations which usually led to the formation of papules or ulcers, and third, lesions of a granulomatous type consisting either of circumscribed masses or of more diffuse granulomatous processes.

The acute inflammatory process which marked the beginning of many of these localized infections was a fairly characteristic feature of affections of the moist surfaces or the orifice of the sheath. In one group of cases, there was a diffuse redness and swelling of the parts as in Fig. 18, which lasted for only a short time as a rule and was superseded by a diffuse infiltration (Fig. 19). As the process advanced, erosions or definite ulcers made their appearance in various localities but more especially about the margins of the sheath. Lesions on the skin surface were covered by dry, adherent crusts, while those on the mucous surfaces of the sheath and the shaft of the penis were moist and covered by a gray necrotic exudate. Occasionally small papules or condylomatous growths were also present (Fig. 18).

In another group of cases, the initial lesion was of a more circumscribed character and appeared in the form of a slightly elevated area of a gray, pink, or amber color surrounded by a zone of slight inflammatory reaction (Figs. 20 and 22). Some of these processes tended to spread and formed diffuse areas of infiltration (Figs. 20 and 21), while others developed into circumscribed and indurated nodules as was the case with the lesion shown in Figs. 22 and 23.

When the infection was confined to the skin surface, the acute inflammatory reaction was usually slight and the conditions present were much the same as in the case of other skin lesions. Thus in Fig. 24 there is seen an area of infiltration involving the right side of the sheath which is just beginning to show surface necrosis and eventually developed into the chancre-like mass seen in Fig. 25.

This type of affection was comparatively common and presented numerous variations of the condition illustrated. Two especially marked cases of infection confined to the region of the meatus of the sheath are shown in Figs. 26 and 27. It will be seen that the lesions completely surround the orifice of the sheath and are located practically at the line of junction of the skin and mucous surfaces. In one case (Fig. 26), the necrosis appeared exactly along this line and completely encircled the meatus. In the other (Fig. 27), the necrotic area occupied a similar position, but at the time the photograph was taken, the ulcer was confined to one side.

Another form of unusually marked involvement of the sheath is that shown in Figs. 28 and 29. The condition began with edema and congestion of the sheath, and, as the lesions developed, there was marked enlargement and induration of the sheath extending from meatus to base, associated with a pronounced exfoliation of surface epithelium and the formation of cracks and erosions of the skin

and mucous surfaces (Fig. 28). Eventually, an irregular line of necrosis and ulceration appeared and separated the mucous from the skin surfaces. On the whole, the mucous membrane appeared to be less involved than the skin, and no definite involvement of the penis could be made out.

Involvement of the penis, as has been mentioned, may occur along with that of the mucous surface of the sheath, in which case the lesions seen were of the type of small infiltrations or surface erosions. One other form of lesion may be mentioned which was observed in but one animal. This consisted of an elevated area of infiltration surrounding the urethral orifice as is indistinctly shown in Fig. 30. In addition, it may not be out of place to mention that infection of the mucous membrane of the urethra with the production of lesions has been demonstrated microscopically.

Occurrence and Duration.—As might be expected from their proximity to the point of inoculation, lesions of the sheath developed somewhat earlier than those of the other mucocutaneous surfaces. The earliest lesions of this group appeared 26 days after inoculation, in other words, almost as soon as the primary lesion itself. There were two other cases which occurred within 2 months after inoculation, and a number of cases within a period of 3 months, while the latest case of the series was 6 months after inoculation. The subsequent history of lesions of this group was very variable. Some lasted for a comparatively short time, while others endured for many months. For example, the lesions shown in Figs. 28 and 29 were still fairly active when the animal was killed for pathological examination $5\frac{1}{2}$ months after the lesions first appeared, and in a second animal, the lesions persisted for a little more than a year.

Affections of the Anal Region.

With a few exceptions, animals which showed specific involvement of the sheath showed lesions of the anus as well, and, conversely, there were only two animals with anal lesions in which corresponding lesions were not present on the sheath. The two groups of conditions, therefore, might almost be considered as one, and apparently the main difference between them was in the character of the lesion which occurs in the two locations, and even this was not great. There were no true exudative affections of the anus. The lesions situated on the cutaneous surfaces were essentially the same in all respects as those

of the sheath. In fact, there was a very striking similarity between the lesions present in the two localities, as may be seen by comparing those shown in Figs. 24 to 29.

The most interesting conditions seen by us in the anal region of the rabbit were lesions which might be classed as condylomata, a typical example of which is given in Fig. 31. These lesions were, as a rule, entirely obscured from observation until the anal ring was distended to a sufficient extent to permit of inspection of the transitional borders. This could be easily done by exerting pressure upon the rectum immediately behind the sphincter.

The first suggestions of the presence of lesions of this character (condylomata) came with slight redness and swelling of the anus, and during the earlier stages of the localized infection, no other alteration might be present. As infection advanced, the reaction became more localized, with the development of areas of infiltration and induration situated in one or more segments of the anal ring immediately along the junction of the skin and the mucous membrane. These were not unlike the patches of infiltration on the mucous surfaces of the sheath. In cases of more pronounced involvement, however, such as that shown in Fig. 31, the entire anal ring was involved, with extension of the process over both the mucous and cutaneous surfaces. The lesion thus formed was a distinctly elevated and indurated mass strikingly like the condyloma latum in man. Judging from our small series of animals, lesions of the condyloma type are more frequent about the anus of the rabbit than on the sheath, and this constituted the chief difference between the lesions of the two localities.

Occurrence and Duration.—As regards the time of occurrence and duration of anal lesions, they are again comparable to those of the sheath, but, on the whole, appeared to be slightly more delayed in their development and in a few instances were more enduring. The condyloma shown in Fig. 31 remained active for upwards of 15 months and still showed slight signs of activity when the animal was killed 21 months after inoculation.

Diagnosis of Mucocutaneous Infections.

Obviously there are many conditions affecting the rabbit which might be difficult to differentiate clinically from some of the affections described above. This is especially true of infections of the nose, eyes, and genitalia, but rare in the case of the anus. A syphilitic rhinitis which is not associated with characteristic lesions presents much the same train of symptoms as the more common condition

known as snuffles. The chief symptomatic difference between the two affections is in the associated involvement of the lacrimal system. As a rule, snuffles is not associated with marked and persistent lacrimal overflow, and when involvement of the lacrimal system does occur, there is usually an acute conjunctivitis. On the other hand, profuse lacrimation without an acute inflammatory reaction is very common even in the early stages of a syphilitic infection. At this stage, however, the distinction between the two processes can be made with certainty only by a demonstration of spirochetes in the nasal discharge or in the mucous membranes themselves.

In cases in which lesions are present, the differentiation of the two conditions presents less difficulty. In the most severe and long standing cases of snuffles, some infiltration and erosion may be present about the nares but they are associated with a greater degree of suppuration and could hardly be confused with the conditions described above.

The two main conditions to be considered in connection with the eyelids and conjunctiva are trauma and conjunctivitis of bacterial origin. Slight abrasions of the lids are fairly common among rabbits, especially as a result of scratching, but they usually heal very quickly and should not be confused with syphilitic infiltrations. There is also a parasitic disease of the skin (mange) which occasionally forms small lesions along the margins of the lids, but these are readily identified by the character of the lesion and the presence of a similar affection upon other parts of the body.

Traumatic conjunctivitis and epizootic infections of the conjunctiva must also be distinguished from affections of a syphilitic nature. These conditions, however, have a more immediate connection with affections of the eyes than with those of the present group.

There are several conditions, chiefly infections, which should be mentioned as possible sources of confusion with syphilitic involvement of the penis and sheath. So called gleet is a well known disease of rabbits which affects the genitalia of both males and females and is characterized by an acute inflammatory reaction with a purulent exudate and may lead to necrosis and ulceration. We have seen a few such cases among normal animals but know very little of this condition from personal observation.

Arzt and Kerl (6) have described a similar condition in rabbits due to a spirochete infection which is capable of transmission from one animal to another. It is claimed that the lesions produced by this organism bear some resemblance to syphilitic lesions. We have never encountered infections of this type.

Traumatic and pyogenic infection about the genitalia must also be considered, but as far as we are aware, the only class of syphilitic affections which might be confused with any of the conditions mentioned are those in which no characteristic lesions are present, and in these cases, a diagnosis can be made only by the demonstration of spirochetes.

SUMMARY.

In a series of more than 200 rabbits in which generalized lesions were observed following local inoculation with *Treponema pallidum*, there were a number of animals in which characteristic lesions were noted upon mucous membranes or along mucocutaneous borders. These lesions were distributed with about equal frequency between the nose or nasolacrimal system and the eyelids on the one hand, and the genital and anal regions on the other. The lips and buccal mucosa appeared to be less subject to localized infections unless the papillomatous growths noted on the lips and under side of the tongue should prove to be in some way connected with such an infection.

In many instances, the local reaction was initiated by an acute inflammatory process, and in the case of nasal and genital infections, a definite exudate was formed. The succeeding stages of the reaction consisted in an infiltration of the parts involved, together with a variable degree of proliferation of fixed tissue cells, leading eventually to necrosis and ulceration. The resulting lesions differed according to their location and the character of the reaction in the individual case. Localized infections of the nose occurred in several forms, first, as a rather diffuse affection of the nasal mucosa characterized by the presence of a mucopurulent exudate, second, as a more or less circumscribed process of infiltration with an especial predilection for the region of the anterior nares, and third, as a granulomatous process involving the alæ in particular.

Involvement of the nasal mucosa was very commonly associated with lacrimal overflow and with some degree of conjunctivitis.

The lesions of the eyelids were usually small, elevated papules or lesions of an ulcerative character some of which were surrounded by a zone of infiltration. In exceptional instances, large granulomatous lesions occurred along the margins of the lower lids.

Infection of the penis and sheath gave rise to conditions analogous to those of the nose. In one group of animals, there was a diffuse affection characterized by redness and swelling of the parts with a mucopurulent exudate, in another there were circumscribed or diffuse infiltrations, while in a third the lesions formed were indurated granulomatous masses. Secondary necrosis with erosion or ulceration was a common feature of all these conditions.

Localized infections in the region of the anus differed from those in other localities chiefly in the absence of an exudative group of affections and in the frequency with which lesions of a papillomatous type occurred.

Lesions of mucous membranes and mucocutaneous borders developed at periods of time varying from a few weeks to several months after inoculation. Most of them were rather enduring and in several instances persisted in an active condition for considerably more than a year.

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EXPLANATION OF PLATES.

The illustrations are reproductions of unretouched photographs which, with the exception of Fig. 3, represent the objects at their natural size. Fig. 3 is at a magnification of 1.5. Statements of time are estimated from the date of inoculation unless otherwise indicated.

PLATE 78.

FIGS. 1 to 4. Syphilitic affections of the nasolabial region.

FIG. 1. 4 months. Syphilitic rhinitis with infiltration about the nares. There is loss of hair over the affected area, with superficial necrosis and ulceration and the formation of adherent crusts.

FIG. 2. 10 months. Marked infiltration of the alæ with necrosis and the formation of ulcers covered by crusts. There were also pronounced lesions at the angles of the nares.

FIG. 3. 68 days. A circumscribed and elevated area of infiltration with central necrosis and ulceration situated in the right nasal orifice. The lip is drawn down to bring the lesion into view.

FIG. 4. 5½ months. A marked diffuse infiltration of the skin and mucous membrane of the left side of the nose and the nasolabial fold with slight involvement on the right. The skin area shows exfoliation of surface epithelium and there were erosions on the mucous membrane.

PLATE 79.

FIGS. 5 to 8. Stages in the progress of an ulcerating granulomatous lesion of the nose.

FIG. 5. 4½ months. Early granulomatous lesions in the anterior nares with an ulcer on the right. There was no nasal discharge at this time.

FIG. 6. 2 weeks later. A slightly later stage of the affection showing the marked prominence of the alæ and the extension of the necrosis and ulceration, now present on both sides.

FIG. 7. 7 months after the appearance of the lesions. There has been marked destruction of the soft tissues of the nose, and both nostrils are occluded by thick reddish brown crusts.

FIG. 8. The same lesions 1 week later with the crusts removed. There was still slight activity in some parts of the lesions most noticeable on the right. The left nasal orifice was entirely obliterated, and there was only a small opening on the right.

PLATE 80.

FIGS. 9 to 11. The clinical appearance presented by animals showing a combined nasolacrimal involvement.

FIG. 9. 3 months. The same animal as that in Fig. 1, showing an earlier stage of the affection. Note the characteristic mucopurulent discharge about the nose associated with lacrimal overflow. This condition is not unlike that sometimes seen in snuffles.

FIG. 10. 5 months. The same animal as that in Figs. 5 to 8. A pronounced lacrimal overflow was present in this animal without an associated nasal discharge. Note the swollen condition of the lids and skin at the anterior or internal angle of the eye.

FIG. 11. The same animal at a later period of the infection (1 year after inoculation). Chronic dacryocystitis.

PLATE 81.

FIGS. 12 to 14. Affections of the labial cleft.

FIG. 12. The appearance of the surface of the labial cleft in a normal rabbit.

FIG. 13. 3 months. A diffuse infiltration of the skin and adjacent mucous surfaces of the labial cleft, more marked on the left than on the right. The skin is denuded of hair on both sides, and the left lip shows a series of irregular ridges covered by gray necrotic epithelium. Mucous patches.

FIG. 14. 3½ months. A sharply circumscribed and indurated ulcer on the inner surface of the left upper lip.

FIGS. 15 to 17. Lesions of the margins of the lids.

FIG. 15. 2½ months. An early papular lesion arising in the margin of the lower lid.

FIG. 16. 3 months. The lower lid everted to show the inflammatory reaction on the conjunctival surface resulting from a syphilitic lesion on the lid.

FIG. 17. 6 months. An indurated ulcer on the upper lid and congestion of the conjunctival vessels.

PLATE 82.

FIGS. 18 to 25. Affections of the penis, sheath, and anus.

FIG. 18. 4½ months. Diffuse infiltration of the sheath with edema and congestion of both the penis and sheath. There are small papular lesions on the sheath and an area of erosion at the superior margin of the fold in the sheath.

FIG. 19. 145 days. A later stage of the lesions in Fig. 18 showing diffuse infiltration and thickening of the sheath.

FIG. 20. 47 days. A small circumscribed area of infiltration on the mucous surface of the sheath with a diffuse redness and swelling. Sheath retracted.

FIG. 21. 2 weeks later. The same animal. The infiltration has become more pronounced and retraction of the sheath is difficult. Congestion and edema have subsided.

FIG. 22. 2½ months. An early papule situated in the transitional area between the skin and mucous surfaces of the sheath. The papule is surrounded by a slight zone of acute inflammatory reaction.

FIG. 23. 2 weeks later. The same animal. The lesion on the sheath has developed into an indurated nodule with central necrosis and ulceration. The edges of the ulcer are inverted and there are marked vascularization and redness of the tissues at its base (mucous surface).

FIG. 24. 3 months. An irregular area of infiltration in the skin of the sheath. The surface of the lesion shows beginning necrosis at two points. On the dorsal surface of the anus, there is also an indurated granulomatous lesion with a depressed ulcer at the center.

FIG. 25. 1 week later. The same animal. Both lesions have increased somewhat and are now of essentially the same character.

PLATE 83.

FIGS. 26 to 31. Affections of the penis, sheath, and anus.

FIG. 26. 3 months. Syphilitic lesions of the anus and sheath situated at the mucocutaneous borders and completely encircling these parts. The lesions were characterized by an intense induration and the development of a line of necrosis which practically coincided with the transitional area.

FIG. 27. 3 months. A similar group of lesions in another animal. The lesion on the anus, however, was confined to one side.

FIG. 28. 3 months. A diffuse infiltrative process involving the entire sheath and anal ring. The surface of the lesion was marked by irregular areas of superficial necrosis and exfoliation; there were also erosions on the mucous membranes.

FIG. 29. 1 month later. The same animal. Note the appearance of a line of necrosis separating the swollen and everted mucous membrane from the skin of the sheath. There was a similar condition of the anal ring.

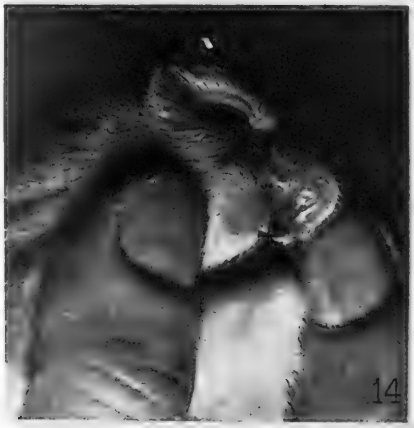
FIG. 30. 2 months. A small mass is seen on the under surface of the penis (marked by an arrow), which was formed by a zone of infiltration surrounding the meatus of the urethra.

FIG. 31. 6 months. Condyloma latum of the anus. The anus is everted.

(Brown and Pearce: Experimental syphilis in the rabbit. V.)

(Brown and Pearce: Experimental syphilis in the rabbit. V.)

(Brown and Pearce: Experimental syphilis in the rabbit. V.)



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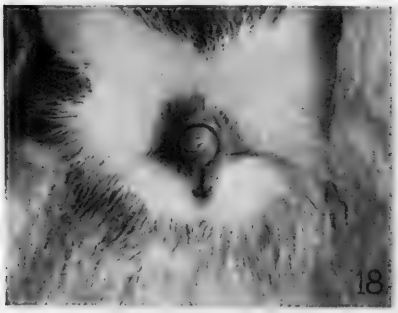
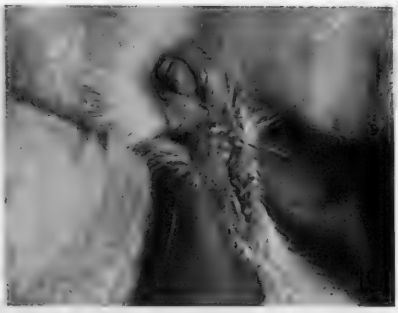
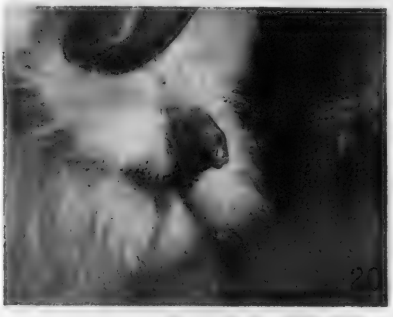
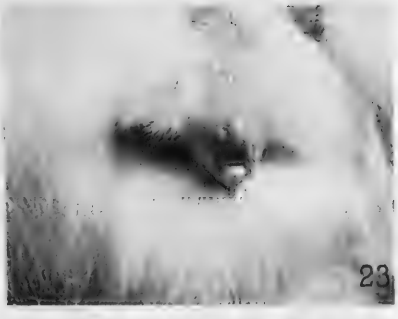
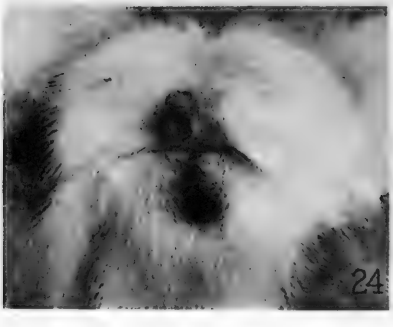
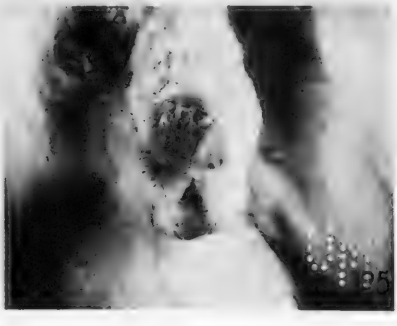




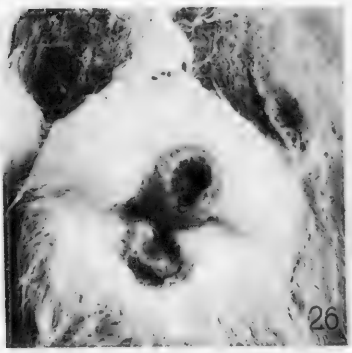


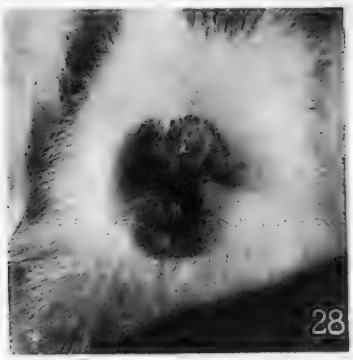


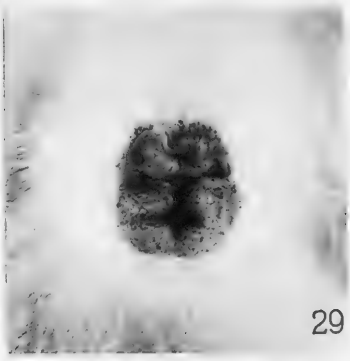


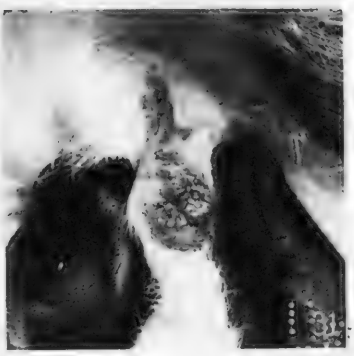


(Brown and Pearce: Experimental syphilis in the rabbit. V.)









(Brown and Pearce: Experimental syphilis in the rabbit. V.)

STUDIES ON ENDOTHELIAL REACTIONS.

II. The Endothelial Cell in Experimental Tuberculosis.

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Plates 84 to 86.

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In the first paper of this series (Foot, 1919) it was shown that the macrophages of the loose connective tissue of the rabbit are of endothelial origin, that they are not derived from the cells of the omentum, the fibroblast, or the lymphocyte, that they are probably chiefly produced by the proliferation of the vascular endothelium in the immediate vicinity of the lesion which calls them forth, and, lastly, that they do not appear to come from distant parts of the body by way of the blood stream, as has been suggested by some authors. The means employed in tracing them to their source was a lampblack gelatin colloidal suspension, or sol, prepared according to the formula of McJunkin and injected intravenously; the agent used to call them forth was sterile melted agar injected subcutaneously.

After studying the effect of so inert and simple an excitant as this, it is natural to turn one's attention to the more complex conditions brought about by microorganisms which are known or believed to cause a somewhat similar reaction, limited chiefly to the endothelium, but probably more variable than that already studied. For this purpose the reaction to the tubercle bacillus was first chosen; that to other organisms will be the subject of future study.

There has been much dispute concerning the origin of the component cells of the tubercle for many years. The epithelioid cell is said by some to be of endothelial origin, by others of purely connective tissue or fibroblastic parentage, while a third group of investigators traces them to a lymphocytic source. There are also some authorities who would admit epithelial and mesothelial cells to this category

of antecedent cell, feeling that the tubercle represents no one cell group alone, but rather a composite structure. This phase of the subject is discussed at length below. For the present we must consider whether the question can be answered by means of intravital staining.

The work of Goldmann, and Evans, Bowman, and Winternitz has shown it to be possible in the case of the liver, where they have demonstrated that the tubercles are formed by the proliferation of the Kupffer cells of the sinusoidal endothelium, using benzidine dyes to stain these cells intravitally and then producing tubercles by injecting tubercle bacilli directly into the portal system. The tubercles were vitally stained in every case. Oppenheimer obtained similar results in 1908, using collargol as the coloring matter.

It seems, therefore, that the origin of the tubercle from the endothelium is proved in the case of the liver. Kupffer cells, however, take up benzidine dyes very well, whereas the endothelium of the smaller capillaries elsewhere takes up little or none, so that it is necessary to choose a more specifically endothelial vital stain before it can be decided whether the tubercles of other organs are also of endothelial origin. It is the more important to decide this question since Sewell, using vital staining methods, has recently claimed that the alveolar epithelium is the chief source of the epithelioid cells of the pulmonary tubercle. Intravenous injections of a suspension of carbon, which must be in the colloidal form, will, as McJunkin (1918, 1919) has shown, serve as a vital stain nearly, if not quite specific for the entire endothelium of the smaller capillaries, particularly if they are subjected to inflammation or injury. It seems advisable, therefore, to produce tubercles in various parts of the body, and, by means of a combination of two or more of these vital stains, to mark the endothelial cell so as to trace it, if possible, to the lesions thus formed.

Before describing the work which I have done along these lines and the results obtained, it would be well critically to consider the specificity of a carbon suspension for the endothelium. It was found that if 5 cc. of a colloidal suspension of lampblack and gelatin, or of equal parts of Higgins' water-proof India ink and distilled water, in which acacia replaces the gelatin, are injected into the ear vein of a rabbit, the veins of the opposite ear become black in about 7 seconds; in a minute's time they resume their normal red color and stay red

until another injection is given. If the animal is killed after 45 minutes, the spleen, liver, and bone marrow are found to be almost black and the lungs definitely sooty; the spleen oozes ink on pressure. Microscopically the spleen shows much carbon free in the sinuses, but comparatively little is intracellular; later it is found to be taken up by the pulp cells, and other endothelial cells. The Kupffer cells of the liver are completely engorged with carbon after 45 minutes, their stellate processes being outlined by the particles of pigment. In the lungs many of the endothelial cells of the alveolar capillaries are filled with carbon and are seen to migrate into the air sacs. The kidney may show some carbon in the endothelium of the glomerular tufts, but none in the epithelium. A large percentage of mononuclear cells in the circulating blood contains the pigment, as may be demonstrated in films or in cross-sections of the larger vessels in various organs. A small amount of free pigment is found in the blood, but this disappears after 24 hours. McJunkin has found that the mononuclear cells of the circulating blood require from $\frac{1}{2}$ to 2 hours to become saturated with lampblack, and he has demonstrated that such cells will phagocytose carbon in large quantities *in vitro* within an hour, both in the case of animal and human blood. This author states that, after injection subcutaneously, no carbon is taken up by the fixed cells of the connective tissue within 2 hours, a fact which he thinks would prove that the phagocytic cells are not of connective tissue origin.

Experimental Subcutaneous Tuberculosis.

In order to test the validity of this seemingly sweeping claim and at the same time to study the earliest reactions to the tubercle bacilli when injected subcutaneously, three rabbits were thus injected with a mixture of tubercle bacilli and Higgins' ink, diluted with equal parts of distilled water, and the resulting lesions studied. It will be seen that the experiment was so arranged as to give a very even series of reactions ranging from 1 to 30 hours. In this way it is possible to follow not only the process of phagocytosis, but also the early reaction to the tubercle bacillus.

Experiment 1.—Three rabbits were given injections at hourly intervals so that a series of lesions would be obtained representing a lapse of from 1 to 30

hours. The injections were made into the subcutaneous tissue of the back, starting at the nape of the neck and continuing toward the tail. They were charted and the lesions numbered for identification.

The amount injected each time was 1 cc. of a mixture consisting of 10 cc. of normal salt solution, two loops of a culture of bovine tubercle bacilli on glycerol agar, and ten drops of Higgins' ink. This combination makes a very even colloidal suspension and gives results perfectly analogous to those obtained with lampblack gelatin. The preparation and sterilization of the latter are thus avoided, as there is enough camphor present to keep the ink sterile. The bacilli were removed from the culture tube and rubbed up in a drop or two of the salt solution with a flat platinum spatula until a cream resulted. Further comminution was obtained by bending the spatula into a J and spinning it rapidly in the cream with the thumb and forefinger. The resulting "mixture" was then diluted up to 10 cc., the ink added, and the whole was shaken for ½ hour in a mechanical shaker, the agitation being repeated before each injection. In this way a reasonably good suspension of the bacilli was obtained. After each animal had had nine or ten injections it was killed by forcing Zenker's fluid into the beating heart under an anesthetic, a large syringe and needle being used for the purpose. The animal was skinned, the lesions were dissected out and spread on paper, and then each lesion was dropped into a separate bottle of Zenker's fluid and fixed for 12 hours.

The material was embedded in paraffin and cut as thin as possible; it cut best when the sections were made at right angles to the flat surface of the lesion; those cut parallel with it were found to be unsuitable for very thin sections, but it was possible to make fair preparations of 10 to 15 microns in thickness, which was always done. They were stained by various methods: Mallory's eosin-methylene blue, phosphotungstic hematoxylin, and connective tissue stain; Van Gieson's connective tissue stain; and various combinations of hematoxylin and carbolfuchsin.

Lesions were obtained chiefly in the subcutaneous tissues, but several were made a trifle deeper into the muscle, and in one case a lymph node was included in the experimental field, the injection missing it by only a millimeter or so.

In describing the results of the experiment, no attempt will be made to itemize the lesions by hours, as it would be unnecessary. An outline of the changes noted will be given with ample indications of the time factors involved in each.

Changes Found after 1 to 7 Hours.—The first change wrought at the site of the injection is an extensive destruction of the subcutaneous tissue, which is forced apart and the cells of which are separated and badly damaged both mechanically and by the action of the large number of bacilli introduced; at first the mechanical injury pre-

dominates. The ink is distributed in a clinging layer over the coarser fibers and on the surface of the cells present, and remains extracellular for a time. Within the 1st hour little if any of it is taken up by the macrophages and fibroblasts, but after 2 hours it can be found within the cytoplasm and surrounding the nuclei of both of these. In the latter, however, it seems to be taken up in a finer form, and to a much less extent, as though these cells were incapable, or had lost their power of phagocytosing coarser particles; in the macrophages it is in a coarser form. Polymorphonuclear leucocytes appear in large numbers at the end of 2 hours and are found throughout the succeeding stages of the experiment. It is noteworthy that one can find an almost immediate response in the endothelium of the smallest capillaries; at the end of 1 hour the cells of their walls are swollen and increased in numbers, in some cases almost blocking the lumina of the vessels. Near these vessels are found a few migrating mononuclear cells of the type described in the first paper, ovoid cells with bean-shaped nuclei and fairly dense cytoplasm; after 5 hours they are not only markedly increased in numbers, but they are actively phagocytic for the carbon (Figs. 3 and 4). Before 4 hours time they are relatively hard to find, except in the immediate vicinity of the vessels, which are never abundant in the sections; after 5 hours one has no difficulty in observing these cells, and they are fairly evenly distributed through the sections. It is of significance that they should be first found near vessels only. The macrophages, or larger type of these wandering endothelial cells, now show definite degeneration, as do the fibroblasts. By far the largest number of healthy cells are those which have migrated into the field of injury, most of the preexisting tissue cells having become necrotic or at least degenerated.

Changes Found after 8 to 30 Hours.—The process continues up to about 8 hours, when it is noted that the small endothelial wandering cells begin to increase in size from 4.8 to 6.5 microns to from 9.5 to 16 microns. They contain carbon and the eosinophil granules of necrotic polymorphonuclears, as well as vacuoles. Some are of irregular, more or less globose shape, some are fusiform, others are stellate. At this time cells resembling fibroblasts begin to be conspicuous (Fig. 5) whereas heretofore they have been an almost negligible factor; despite this fact it is only after much searching that a very

few mitotic figures can be found in them. They usually contain carbon in an evenly divided, rather fine form. It is also noticeable that, while the original fibroblasts had very little cytoplasm, these later fibroblasts have a good deal. It is very reticular and vacuolated and usually stains black with Van Gieson's stain at this stage.

During the next few hours the small endothelial cells are not so noticeable; their place is taken by numbers of the larger type, which will be called macrophages for convenience. These average 6.5 to 9.5 microns in diameter, but many are extremely large and swollen and measure 24 by 10 to 12 microns. Such cells often contain whole polymorphonuclear leucocytes. The latter are now becoming more and more degenerated in appearance and are clumping to form abscesses. These abscesses, as will be shown later, persist for days. The macrophages show a very slight tendency to clump in groups of three or four, and extended search reveals a few polynucleated cells of this type, young syncytia, or giant cells. There is, however, no tendency to form what could be called a tubercle.

Up to this time the bacilli found are extracellular, they are not being phagocytosed to any extent in the first 18 hours, but lie in small masses, surrounded by a palisade of polymorphonuclears which very occasionally take up one or two bacilli that are possibly dead. The living organisms are hemmed in rather than phagocytosed.

The 14 hour lesion included a small lymph node in its sphere of influence. Although but a millimeter or so distant from the injection of carbon and bacilli, the only change noted is a marked edema of the sinuses and reticular cells and a certain activity of the germinal centers and lymphoblasts in the nodules. The marginal sinuses are distended with fluid and contain many of the smaller wandering endothelial cells, but there is no trace of carbon anywhere in the node. No tubercle bacilli can be demonstrated in its substance. The reticular cells show no particular activity, but many mitoses are to be found in the groups of endothelioid cells of the germinal centers, an observation which may be of interest to those who believe the reticulum to be the parent tissue of the macrophages or histiocytes.

The lymphocytes, which are small cells, are usually absent in practically all the sections of the series; they were found in one lesion only, that of the 9th hour. Here they were present in small numbers,

generally near blood vessels and often surrounded by a delicate network of fibrin. Were the tubercles formed from cells originating in lymphocytes, one would expect to find these cells present in quantities, which is far from being the case.

During the next 4 hours, up to the 18th hour, little change is noted; abscess formation is more marked, but otherwise the processes just outlined continue. The activity of the vascular endothelium continues up to this time and is almost exclusively limited to the smallest capillaries; the vessels which have an adventitia and well organized muscular walls do not appear to share in the process, although the adventitial cells occasionally seem to do so.

Sections made from the lesions of the third animal, representing stages from the 19th to the 28th hour, fit into the scheme which we have been constructing, in logical sequence. By 22 hours one finds that there has been a continued and marked increase in the number of large, fusiform cells resembling fibroblasts. These are surrounded by delicate basketworks of fine collagen fibrils, best seen in the Van Gieson preparations; it is also not uncommon to find the fibrils running very closely over the cells and sometimes through their cytoplasm. These cells do not, however, tend to have the tautly stretched appearance, with fanned out extremities, which is seen in the fibroblasts of rapidly growing fibrous tissue. There is a marked increase in the number of small endothelial wandering cells after the 22 hour lesion has been reached, and the increase is very noticeable up to the 30th hour, possibly indicating a second wave of migration on the part of these cells. Although the preparation representing the 30th hour was made from the first rabbit of the series of three, and the ten preceding it from the third, this section presents a picture which dovetails perfectly into the scheme, showing that the reaction does not differ materially in individuals of this species.

Tubercle bacilli are found at first in small clumps in the abscesses that form at about the 15th hour; definite phagocytosis by a number of endothelial wandering cells is first noted after 27 hours, some of these cells containing five or more bacilli without appearing to suffer materially thereby. After 21 hours the bacilli are no longer limited to smallish clumps, but are scattered broadcast throughout the abscesses.

This experiment would show, therefore, that there is at first a de-

structive action on the part of the bacilli and their toxins, in addition to the mechanical destruction caused by the injection, and that this is followed by a reaction on the part of the neighboring tissues, whereby polymorphonuclear leucocytes migrate and the endothelium of the smallest capillaries swells, proliferates, and sends out migratory cells. The polymorphonuclears surround the bacilli and form local abscesses; the mononuclears take up the carbon and later the bacilli, increase four or five diameters in size, and either wander about, or become involved in the formation of fibrils, or degenerate and die. They are actively phagocytic for the debris of the tissue and leucocytes, not only in their wandering phases, but also after they have become fusiform and apparently sessile.

The experiment also demonstrates that the cells of the tissue which has been injected with a mixture of ink and bacilli do not actively phagocytose the carbon contained in the ink, but do so only to a limited degree and then degenerate. Most of the phagocytosis is carried on by cells which wander in after 4 or 5 hours have elapsed. This bears out McJunkin's findings, but it would seem that he overstates slightly when he says that there is no phagocytosis on the part of the sessile tissue cells within 2 hours. It would appear, therefore, that one is justified in stating that, with the exception of the polymorphonuclear leucocyte and a very small percentage of original fibroblasts and their scanty progeny, practically any cell found to contain carbon particles, up to 30 hours after the intravenous injection of water-proof ink, whether intravascularly or extravascularly situated, must be considered as of probable endothelial origin. The polymorphonuclears occasionally phagocytose the carbon, but not so often as one would expect.

In the foregoing description it was shown that there were very few fibroblasts left alive after the first 2 hours following the injection of the bacilli and ink, and that cells closely resembling them began to be evident again after the migrating endothelial cells had begun to increase in size, about the 13th hour. This is in spite of the fact that extremely few mitotic figures are to be found in the surviving fibroblasts. Whence do these new cells come? The most logical supposition is that they are derived from the wandering cells of endothelial origin, particularly as they all contain large amounts of carbon and

some of them whole leucocytes or their eosinophil granules. Of course, some may be produced by the proliferation of the surviving connective tissue cells, but there is comparatively little evidence upon which to base such a conclusion. On the other hand, we can see the small endothelial cell leave the vessel, where the lining cells are swollen and increased in numbers (mitotic figures can be found in these after some searching; they were observed more frequently in the case of some guinea pigs that were experimented upon); we can see them gradually becoming filled with carbon particles and increasing in size (Fig. 1); we can demonstrate numbers of them in spindle-shaped phases, many of them in stellate or irregularly ameboid outlines; and then we find these "fibroblasts," filled with carbon and fitting perfectly into the transition picture. This transformation is stressed here, because it must be more thoroughly worked out at some future time. Of course, the cells were stained with the usual connective tissue dyes before the conclusions just indicated were reached.

The subcutaneous reactions to the tubercle bacillus were also studied from another angle, the bacilli being injected subcutaneously without any pigmented mixture. Eight rabbits were used in this experiment, the ink being given intravenously and the subcutaneous lesions produced forming a graded series, as in the first experiment. The animals were treated as follows:

Experiment 2.—Each animal received three injections of 10 cc. of 1 per cent aqueous solution of trypan blue or red intraperitoneally, at daily intervals, and then one injection every 3 days during the rest of the experiment. One loop of stock culture of bovine tubercle bacilli was suspended in 10 cc. of salt solution as in the other experiments and 1 cc. injected under the skin of the back. In two rabbits a few milligrams of carmine rubrum optimum was mixed with the suspension, but this was discontinued as superfluous in the later experiments. The injections were repeated at varying intervals, in the case of the last four rabbits once a week, and spaced down the back so as to produce five or six graded lesions between neck and tail. At first 2 to 3 cc. of Higgins' ink and distilled water, in equal parts, were injected intravenously every 3rd or 4th day, but the rabbit that survived the longest had the dose increased to 5 cc. for several doses prior to its death from epizootic pneumonia.

Most of the rabbits were killed by injecting neutral 4 per cent formaldehyde into the beating heart, under anesthesia, or were autopsied as soon after death as practicable, in which case the formaldehyde was also injected into the heart under pressure. The tissues were fixed in the same fluid and were placed in

Zenker's fluid after being sectioned in paraffin whenever this was necessary for the staining reactions. The slides were incubated for 2 or 3 hours in Zenker's fluid at 62°C. Paraffin sections were stained with Delafield's hematoxylin with or without carbolfuchsin, with Van Gieson's stain with or without carbolfuchsin, and with Mallory's connective tissue stain and his phosphotungstic acid hematoxylin. The fixation was admittedly not the proper one for the last two methods, but fair results were, nevertheless, obtained.

In the first experiment the changes that took place in the first 30 hours were observed; this one begins where the other left off and continues up to 49 days. In the first experiment there was little or no evidence of true tubercle formation, at best a slight tendency to clumping was exhibited by the wandering cells. In the series now under discussion the reaction was found to be invariably the same, the formation of an abscess with small tubercles scattered around its periphery. These are engulfed in the advancing caseous abscess and new ones form at the constantly enlarging border. For this reason and with this heavy dosage it was impossible to obtain anything resembling the graded series of the first experiment, or that in which agar was used as an irritant. On the 5th day the small peripheral tubercles begin to form and spread into the outlying tissue; any lesion older than this merely shows the same condition on a wider scale. Thus we are limited to the study of these small tubercles, which are typical and range from submiliary to confluent in nature. There is no appreciable difference to be noted in the lesions from one or another animal of the series, except for the variation in the amount of carbon and vital dye contained, which is readily explained by the fact that some of the animals were killed soon after, others a day or more after the last injection of ink or dye. Repeated injections of tubercle bacilli seemed to cause no variation in the type of lesion produced; the later ones differed in no respect from the earlier ones. In two of the rabbits neighboring lymph nodes became infected through the lymphatics, and tiny tubercles, without much caseation, were produced in them.

The true tubercles, best studied after 2 weeks of development, on account of their greater numbers, consist of large mononuclear cells, liberally sprinkled with carbon particles, and of lymphocytes, the latter always free from this pigment. The endothelium of the ves-

sels in the invaded tissue always shows plentiful carbon, in some cells sparsely scattered, in others so liberally distributed as to obscure the cell anatomy. Sometimes the mononuclear cells are found free in the lumina of these capillaries, containing varying amounts of carbon, but they are seldom found in the older preparations, in the circulating blood of the larger vessels. So striking is the distribution of carbon in the tubercles that one can glance over a slide with a low power objective and immediately locate the lesions by reason of their dusky color. In one of the animals in which trypan red was used, the tubercles were not only dusted with carbon but were also stained brick-red.

In giving a detailed account of the relation of the coloring matter to the cells it will be necessary to take up each substance in turn. The carbon is found almost invariably in mononuclear cells; little is taken up by the polymorphonuclears, although no citrate was combined with the injection. These cells are found (*a*) free in the circulating blood near or in the lesions, (*b*) in the endothelium of the blood capillaries, seldom in that of the lymphatics, (*c*) in the epithelioid cells forming the tubercles, where the carbon is usually in a fine, more or less separated form, although it may be found very liberally distributed as shown in Fig. 2, and (*d*) in the syncytia, or giant cells, formed by the fusion of these cells, as would be expected from what has already been described in the experiments with agar injections.

It would have been very difficult to trace the carbon from the vessels to the tubercles with only this experiment as a guide; fortunately the first experiment described above makes it clear that this substance is taken up by the cells composing the smallest capillaries and that these cells then migrate and congregate to form the tubercles. The agar experiment, too, makes the mechanism clear. It is probable that the cells of the capillary walls swell and proliferate in response to the action of the bacilli in the neighboring tissue and become more permeable to the carbon, or more sticky, attracting it and then taking it up. Although it was not possible to find many mitotic figures in the rabbit series, they were found with comparative ease in the endothelium of the smaller capillaries in infected guinea pigs which were studied during preliminary experiments, and were easily found in the lungs of rabbits experimented on in a subsequent series. Carbon is also present in cells containing fibrillæ which stain with acid fuch-

sin in the Van Gieson stain and Mallory's connective tissue stain. A few of them show blue fibers with the latter stain.

The trypan dyes are found in the usual sites, mononuclear phagocytes, or macrophages, and connective tissue cells. Trypan red is also occasionally taken up by the polymorphonuclears in lesions of the subcutaneous tissue and lymph nodes, where there is evidence of stagnation in the circulation. It is sparingly found in the capillary walls under like circumstances. Unlike trypan blue it stains collagen fibers diffusely, and like that stain colors necrotic tissue in the same manner. As the mononuclear cells containing it increase in size and take up more bacilli, the dye granules tend to disappear, the stain reappearing diffusely once necrosis has set in. The acid used in differentiating the carbolfuchsin stain (20 per cent nitric) changes the trypan red to a dirty brown and tends to diffuse it throughout the cell, but the granules can still be made out as brownish masses, while trypan blue is diffused, but unaltered in color.

A 1 per cent solution of trypan red turns the same color *in vitro* if 20 per cent nitric acid is added; the original color is restored by neutralizing with ammonia. Trypan blue is only slightly affected by the acid, becoming a little more reddish; the blue is more or less completely restored by neutralization.

In the cases in which powdered carmine is injected with the bacilli, it is found in the phagocytic cells and fills them more or less completely, according to their size and proximity to the pigment; the smaller endothelial wanderers contain a few granules, the larger forms may be quite filled with them. Tubercle bacilli are found in these cells, and one may find them containing granules of benzidine dye, bits of carmine, carbon particles, and bacilli at the same time.

In this experiment, then, the findings of the first are confirmed in that all these substances are phagocytosed by the same cell in the infected areas and that this cell forms the tubercle and is, therefore, the epithelioid cell, and that it originates in the walls of the smallest capillaries and is, for this reason, endothelial. The more one follows out this line of investigation, the more impressed does one become with the fact that the endothelium of vessels whose walls are made up of several layers of cells and fibers seems to be more specialized and, perhaps for this reason, less affected by outside influences than the cells which compose the capillary walls.

That the lymphocytes play a part in the formation of the tubercles is evident, but they appear relatively late, and it is doubtful that this rôle is a formative one; we are thrown back upon hypothesis, as far as they are concerned. There is no evidence from these experiments that they are transformed into the much larger and paler tuberculous epithelioid cell; they do not contain any of the colored substances used and it is difficult to find any intermediate stages that would fit them into such a transition picture. Neither has the lymphoid tissue proper any affinity for the benzidine dyes or carbon; it is only the reticular cells that take them up. This is true of both lymph nodes and spleen.

In this, as in the first experiment, phagocytic cells are found which stain with the ink and also contain bacilli, while they are intimately connected with fibrils which take acid fuchsin intensely (Figs. 6 and 7). True collagen fibrils can be demonstrated in some of them. Both in the case of the reticulum of the tubercle and the collagen fibers which surround the lesion, too dense an accumulation of delicate fibrillæ is found to warrant our considering all of them preexistent; they seem to be newly formed. This is particularly evident in connection with the tubercles in the lymph nodes, where the fibrous reticulum is normally very scanty and delicate, and the fibers which we find seem to be too intimately connected with the carbon-bearing cells not to be in some way related to them. These fibers stain darkly with phosphotungstic acid hematoxylin, blue or brownish blue. The further discussion of these fibrils must be relegated to another paper, for the principles involved are too far reaching to be taken up without thorough investigation.

The lesions in the lymph nodes are, in every particular, similar to those studied in the subcutaneous tissue and the connective tissue of the muscles infected. They are invariably perivascular, and the small blood vessels in or near them show the same proliferative changes in their walls. The cells forming these vessels are indistinguishable from those composing the tubercles; in fact the lumen of the vessel becomes a canal in a mass of tissue composed of homologous cells, most of which show carbon in varying amounts. Syncytia are abundant and usually show many carbon granules. In the normal portions of the nodes little carbon is to be found, save in cells circu-

lating in the blood capillaries; the reticular cells do not ordinarily take up much of it unless included in the lesions.

Comparing the findings of these experiments with those of the first one, in which agar was the exciting agent, we find that the reaction is essentially the same in its inceptive stages, but that it differs from the latter in that it is more than mechanically destructive, that it is progressive, and that the action of the exciting agent (tubercle bacilli) continuing over an indefinite period of time repeatedly frustrates the reparative process. In this way, instead of a mass of simple syncytia and subsequent organization, there is only an attempt at their formation, which is then terminated by the destructive activity of the bacilli, with resulting caseation. Therefore, whether the bacilli are intravascularly or extravascularly situated, tuberculosis is primarily a disease the lesion of which is formed by cells originating in the smallest branches of the vascular system; it will be shown in the next paper (Foot, 1920) that this is also true in the case of experimental pulmonary lesions.

General Discussion of the Literature on the Subject.

The origin of the epithelioid cell has been, as already stated, a constant subject of dispute; the standard text-books of pathology, published in recent years, show that there is still no unanimity in regard to this point. MacCallum gives a very good review of the various theories, in conclusion showing that he is definitely inclined to favor that of Maximow (1906, 1909, 1910), which traces the epithelioid cell to the lymphocyte. Adami and Nichols, and Mallory are in favor of their endothelial origin. Askanazy and Lubarsch, writing for Aschoff's system, take the more conservative stand that the connective tissue, vascular endothelium and epithelium, as well as the mesothelium in some instances, are all responsible for these cells. Delafield and Prudden believe that they have their source in the endothelial and connective tissue cells. One might continue to quote opinions quite as divergent.

The literature on experimental tuberculosis dates back to the early seventies and is rather voluminous; most of the important authorities will be found listed in the references at the end of this paper; for a more complete bibliography the reader is referred to Kockel's article. In the early history of the study of the origin and histogenesis of the tubercle two opposing groups are recognizable, the adherents of Baumgarten (see Kockel), who claimed the cells of the fixed tissues (including the vascular endothelium) as the parents of the epithelioid cell, and the followers of Metschnikoff, who saw in the process an exudative phe-

nomenon, or migration of the mononuclears from the blood stream, with subsequent proliferation of these cells in their new situation. Curiously enough, the earliest observers in 1871 and 1873, were inclined to consider the vascular endothelium alone responsible for these cells; with time technique and theories became more and more elaborate and this view-point was lost, or rejected, by most authorities. Of late it has tended to come more and more into prominence.

The weight of opinion seems to favor the endothelium and fibrous connective tissue as the origin of the epithelioid cell, with the epithelium playing a similar part in the case of the eye, lung, and kidney. That the endothelial cell is its parent in the case of the liver is claimed and, it would seem, proved, by Kockel, Miller, Oppenheimer, Goldmann, and Evans, Bowman, and Winternitz. Watanabe and Sewell are of the opinion that the alveolar epithelium of the lung may be the main source of the epithelioid cells of experimental pulmonary tubercles, while Wechsberg believes the endothelium to be their origin. We shall have occasion to take this up in detail in the next paper, so the subject will not be discussed further here. Kostenitsch and Wolkow, who have worked on the eye, think that both the endothelium and the pigmented epithelium share in the process. The kidney has furnished enough evidence of epithelial proliferation for the same authors to allot a share in the formation of renal tubercles to the tubular epithelium. As to tubercles produced elsewhere in the body, one finds little theory based on experimental evidence.

Theories as to the formation of tuberculous syncytia, or giant cells of the Langerhans type, appear in almost every paper; Lambert's work on the foreign body syncytium *in vitro*, and Forbes' and my own, in connection with its production in response to injections of sterile agar, are probably applicable to this cell in the tubercle. There seems to be nothing particularly specific to tuberculosis in its presence, when its appearance in the gumma and other forms of chronic inflammation are considered.

The most interesting confirmation of the findings of the two sets of experiments described in this paper is to be found in the articles of Miller and Kockel. The former shows that the first reaction to the tubercle bacilli in the liver is an exudate of polymorphonuclear leucocytes, which surround the organisms and arrange themselves in "little heaps." He finds the bacilli phagocytosed for the first time 100 hours after injection, considerably later than when noted in the present experiments. He puts the first appearance of lymphocytes at 6 to 9 days, which corresponds accurately with what has been found in the case of pulmonary lesions (Foot, 1920) so that he, too, would seem to rule these cells out of the earlier reaction.

Kockel experimented with injections of farina into the portal vein and produced pseudotubercles, the formation of which was analogous to that of true tubercles, except that whereas the pseudotubercles became vascularized in from 3 to 4 days, the true tubercles showed no inclination to undergo a similar change. The smaller type of endothelial cell was much more in evidence in the pseudo-

tubercle than in the true tubercle, as is the case if reactions to agar are compared with those to tubercle bacilli. Necrosis was present in the tubercles formed in response to the farina; Kockel argues that this is due to stoppage of vessels, which he found to be thrombosed in many cases, rather than to the action of toxins, as one would suspect in the case of true tubercles. Vissman, however, finds that dead tubercle bacilli, when used in such an experiment, while they produce tubercles, cause no caseation thereof, a fact not in accord with Kockel's theory. Morse reported and demonstrated that tubercles, apparently in every way similar to those produced in the rabbit lung by injection of bacilli, could be formed in response to the injection of waxes extracted from dead cultures of the bacilli. Not only were these tubercles cellular structures, but caseation was also present and widespread. This is at variance with the findings of Vissman. The explanation given by Morse is that the leucocytes are hindered in their phagocytic activity by the presence of the wax.

Kockel states in this connection:

> From the peculiar behavior of the endothelium in the periportal granulation tissue and in the young intracapillary tubercles it may be assumed that caseation is less likely the result of direct action of tubercle bacilli on the cells, than of the inhibition of the formation of new vessels and the obliteration of preformed vessels owing to the extreme proliferation of their endothelium which is the result of this injurious action.

He argues that the endothelium proliferates in tuberculosis, as it would in any granulation tissue, for the purpose of forming new vessels; but, owing to a stoppage in the flow of blood and the *vis a tergo* thereof, this proliferation takes the form of sheets or plugs of cells, which are then known as tubercles. That this theory is largely true is well shown in some of the slides which have been studied in the present work, particularly in the case of the lymph node infections in the second series of experiments; that the absence of the *vis a tergo* of the circulating blood plays as important a part as he would ascribe to it cannot be so readily accepted.

As far as the theories of the different schools are concerned, we have already seen how much evidence there is in favor of the endothelial origin of the epithelioid cell. That Metschnikoff's theory as to the exudate of mononuclears from the blood stream, with subsequent multiplication *in situ*, is at least partially correct is evident when one considers that the carbon-bearing endothelial cells are found in the lumina of vessels in the infected areas. That they come from very distant parts of the body seems unlikely in view of the fact that there is such marked evidence of local proliferation of the endothelium; that they are free in the circulation remains undisputed; and that they may migrate and, in the tubercles, join other endothelial cells,

which left the vessels by the external route, must be admitted. Mitotic figures are easily found in the smaller endothelial cells after they reach the site of inflammation, which would further corroborate Metschnikoff's view, although it cannot be determined whether or not these cells were ever free in the circulation. It seems probable that Metschnikoff's theory is correct, if taken with reservations; such cells as he refers to probably migrate in numbers much smaller than those of the vascular endothelial cells which leave the walls by the external route, and they probably come from the walls of capillaries in the proximity of the lesion, rather than from so distant a point as, for instance, the spleen or bone marrow. There is little to indicate that they come from the lymph nodes, even in the immediate vicinity of the lesion.

That the fibroblast is the parent of the epithelioid cell is not borne out by the evidence which is at hand, although the reverse may be true of the fibroblasts which tend to wall off the lesion, as has been indicated. As to the lymphocyte, it has already been stated that nothing has been found to show that it develops into the larger cell; it is present in the tubercle and therefore is a component thereof, just as is its derivative the plasma cell; but it appears too late to be considered as a potential epithelioid cell. This paper does not deal with the epithelium or the mesothelium; they will be considered as possible factors in later studies. Part of the work has already been completed and it may be said in advance that the epithelial cell may be ruled out in the case of experimental pulmonary tuberculosis as a potential epithelioid cell.

SUMMARY.

1. The epithelioid cell is of definitely endothelial origin.

2. The only reliable means of identifying and tracing this cell is, at the present time, a colloidal suspension of carbon, injected intravenously. Benzidine dyes will not accomplish this if used alone.

3. There is little evidence that the local tissue elements take an active part in the process of tubercle formation, until after the lesion is formed; the reaction is, in a sense, exudative, since the lesion is produced from cells which migrate to the site of inflammation.

4. The lymphocyte appears late and is not to be considered as a potential epithelioid cell; its presence in the tubercles is as yet unexplained.

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EXPLANATION OF PLATES.

PLATE 84.

FIG. 1. Semischematic camera lucida drawing of carbon-containing endothelial cells from the exudate, to show their enlargement over a period of 24 hours. Cells *a* to *d* are the earliest type seen; *e* to *g* can be found after about 8 hours following the injection of bacilli and lampblack suspension. Cell *h* is an extremely large form, seen in the latter part of the series of slides, at about 24 or more hours after injection. × 1,120.

FIG. 2. A tubercle found in the second experiment, with an excessive amount of carbon particles. 42 days after injection. Camera lucida drawing. × 1,120.

PLATE 85.

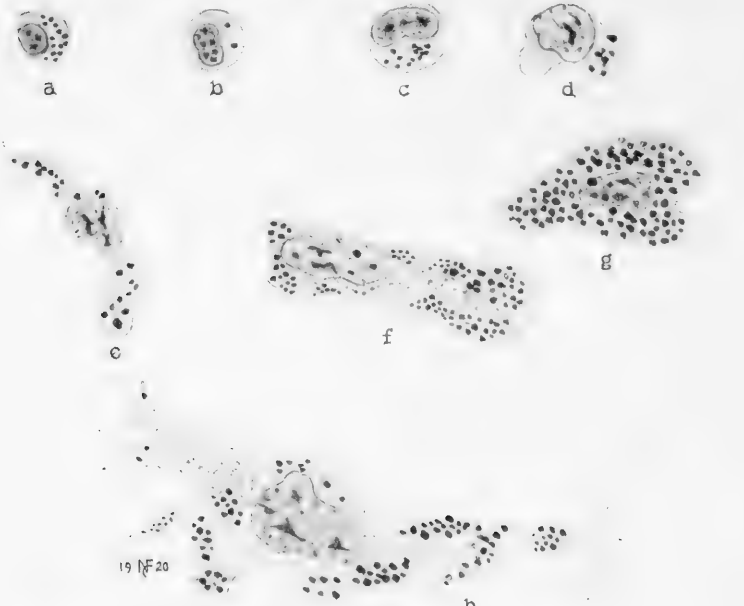
FIG. 3. Photomicrograph of endothelial leucocytes in early exudate. × about 1,000.

FIG. 4. Similar cells in denser tissue. × 1,000.

FIG. 5. Two fusiform types, one burrowing into the dense tissue, with carbon particles in its curved "tail." × about 1,000.

PLATE 86.

FIGS. 6 and 7. From tubercles on the 48th day after injection. Note the distribution of carbon in both, of the reticulum in Fig. 6, and of the bacilli in Fig. 7. × about 800.



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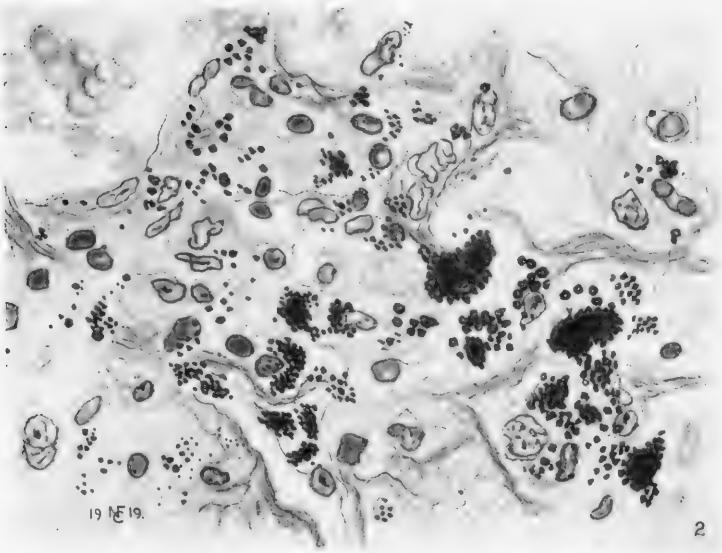

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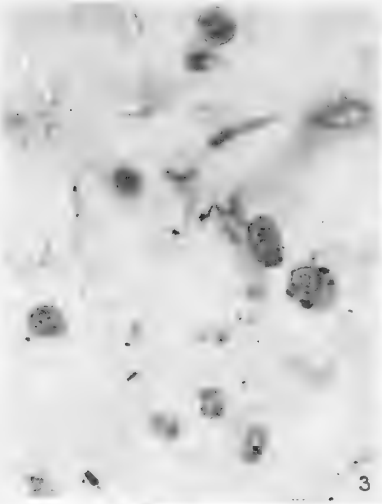
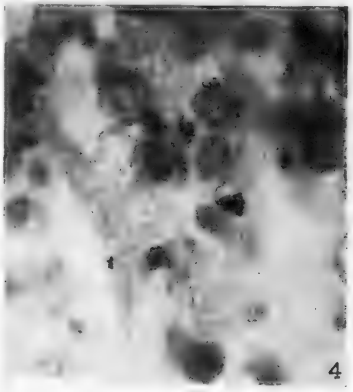
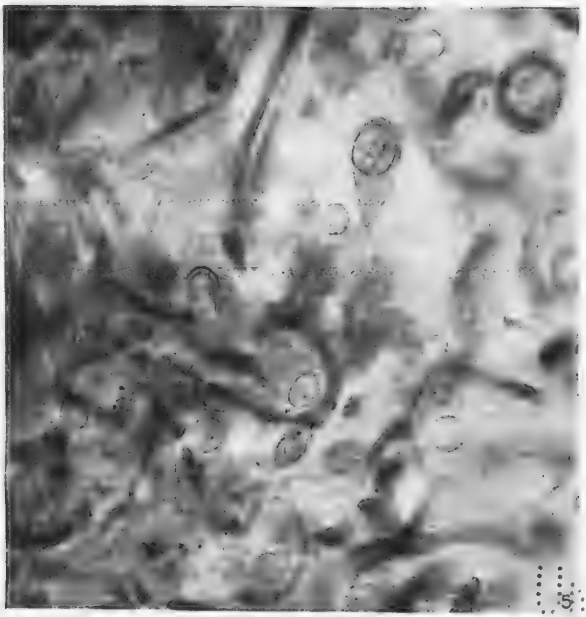
(Foot: Endothelial reactions. II.)







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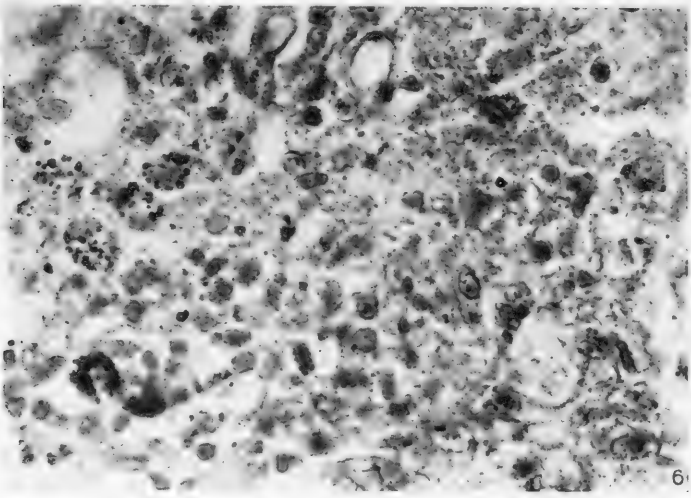

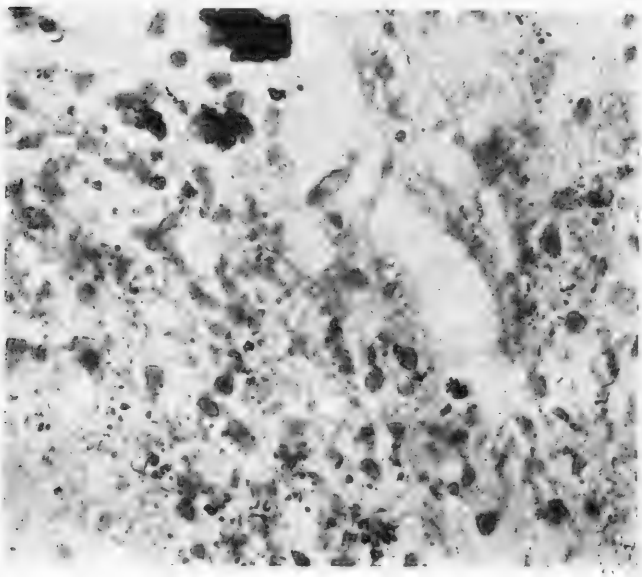

(Foot: Endothelial reactions. II.)

STUDIES ON ENDOTHELIAL REACTIONS.

III. The Endothelium in Experimental Pulmonary Tuberculosis.*

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Plates 87 and 88.

(Received for publication, May 26, 1920.)

In two preceding papers of this series (Foot, 1919, 1920) the reaction of the endothelium to a foreign body, agar, was discussed, as well as its reaction to the tubercle bacillus when introduced subcutaneously. It is the purpose of this communication to consider the reaction to this organism when injected through the trachea into the lungs, with special reference to the origin of the epithelioid cell of the pulmonary tubercles. It has been shown in the second article of this series (Foot, 1920) that it is the endothelial cell which forms this component of the subcutaneous tubercle. Wechsberg, writing in 1901, apparently considers the endothelium to be the source of these cells in the pulmonary tubercle, but both Watanabe and Sewell have taken the stand that the alveolar epithelium plays an important part in their formation. It is in an attempt to answer this question conclusively that the present experiment has been undertaken. A general discussion of the theories on the subject will be found in the second paper (Foot, 1920) and will, therefore, be omitted here. There is strong evidence that the endothelial cell is called out in response to foreign bodies and to the presence of bacteria of a low grade of virulence. That this is true in the case of the liver has already been shown. There does not seem to be any reason why there should be a different response in any other part of the body.

Wechsberg injected tubercle bacilli into the lung by way of the trachea and studied the reactions in sections stained mostly with a view to getting an idea of the damage done to connective tissue. He found that the elastic fibers were destroyed within 6 hours after the injection and that there were defects in the endothelium of the capillaries, through which polymorphonuclears and endothelial cells migrated into the alveoli. He summed up his findings thus: (1)

* An abstract of this article was presented at the 20th annual meeting of the Association of American Pathologists and Bacteriologists, New York, N. Y., April 3, 1920.

There is first a destruction of fixed tissue cells. (2) New formed or migratory cells are injured only in as far as the formation of new vessels is hindered and connective tissue growth is hampered and incomplete. Giant cells are formed. (3) There is a second phase of destruction with caseation. In view of his evidence as to the endothelial origin of the tubercles, it is unfortunate that he does not commit himself more definitely on this head.

Watanabe used the same methods and killed the animals 12, 24, 48, and 96 hours after injection. He found bacilli in "swollen epithelial cells" of the alveolar walls, in the epithelium of the bronchi, and in cells which might be endothelial in origin—"mononuclear leucocytes." He found nothing to indicate that the fibroblast took them up.

Sewell was the first to use vital stains, in the modern sense, in connection with experiments on pulmonary tuberculosis. In his paper he quotes an experiment done by Slavjansky in 1869. This investigator injected suspensions of indigo into the trachea of rabbits and after 2 or 3 days introduced 5 to 7 cc. of a fairly thick "solution" of cinnabar into the jugular vein. 2 days later he killed the animals and found some cells lying free in the alveoli and containing indigo, others containing cinnabar, and a third group free from either. The alveolar epithelium contained indigo and was otherwise unchanged. If, however, he injected the cinnabar into the blood immediately after introducing the indigo into the trachea, he could find cells in the alveoli and bronchial mucosa containing granules of both pigments. He concluded that, in the first case, the cells containing the cinnabar had wandered out of the vessels (thus indicating the possibility of their being of endothelial origin) and finding no indigo free, remained unaffected thereby; while in the second case, they arrived before the supply of indigo had been exhausted and therefore contained both kinds of pigment.

Tchistovitch repeated these experiments in 1889, substituting bacteria for the indigo in the tracheal injection and using carmine intravenously. The animals were killed in 24 hours. Carmine-laden cells were found in the alveoli, and there was also phagocytosis of the bacteria by small cells which contained no carmine granules; he could find no cells containing both, which is not consistent with my findings, as will be seen later.

Sewell injected a suspension of bovine tubercle bacilli in lithium-carmine into the trachea, using 2 cc. at a time. The rabbits were vitally stained with trypan blue and were killed 24 and 72 hours after introducing the bacilli. In one animal he found cells stained with the blue dye lying in the alveoli. As this rabbit showed an extensive pneumonia and pleurisy, he concluded that these cells migrated in response to the infection at a time prior to the injection of the carmine and bacilli, but after the trypan blue staining was complete. Some of these cells contained trypan blue granules, carmine, and tubercle bacilli. The other animal, however, showed only carmine in the free cells. He injected a fine suspension of India ink into the trachea of rabbits vitally stained with lithium-carmine and killed them 3, 6, 15, and 24 hours later. Cells stained with car-

mine did not appear until after 24 hours, but cells containing carbon were found before this and appeared to originate from the epithelium near the alveolar angles. Carmine injected into the trachea gave excellent staining reactions in the alveolar epithelium after 3 hours and trypan blue after 6 hours. He injected 10 cc. of a mechanical suspension of India ink into the ear vein of a rabbit and found none whatever in the lungs.

This investigator did a number of experiments with various dyes injected alone into the trachea of rabbits and guinea pigs, which give an idea of the lesions produced by the injection of such dyes, unmixed with bacteria, while they also serve as valuable control experiments. Quoting Briscoe as to the three possible sources of the mononuclear cells found in the alveoli in pulmonary inflammation, he says that these may be (1) the macrophages in the interstitial tissue near the alveolus, (2) the alveolar epithelium, and (3) specialized epithelium not concerned in respiratory function. His frequent allusions to the similarity of the epithelial and endothelial phagocytes and the fact that he admits that the only means of ruling out the latter in these experiments is the use of intravenous dyes which do not immediately appear in these cells, but require the lapse of a day or more to make their presence known, all indicate the difficulty that is encountered in drawing conclusions from such experiments. Moreover, the carmine that he used intravenously is not specific for the vascular endothelium *in situ* and the mechanical suspension of India ink, upon which he based his final conclusions, did not show in the lungs at all; whereas, had he used a colloidal suspension of carbon, which never fails to show, his conclusions might have been different. In summing up he says: (1) The alveolar cells are endowed with the full phagocytic powers of the histiocyte. (2) Their behavior under the action of a stimulus is similar. (3) When certain dyestuffs are offered to them in colloidal solution, they store up granules of the dye in their protoplasm in the same manner as the histiocytes. He clearly indicates his belief that, in the case of experimental pulmonary tuberculosis, the epithelioid cells are really epithelial cells which are the first to respond to the stimulus of the infection.

Method.

In the experiments which are the basis of this paper I have used two pigments, lithium-carmine and Higgins' water-proof ink, which is a colloidal suspension of carbon in water, with an emulsifying agent and a little camphor. The former was employed solely for intratracheal injections, the latter for the intravenous; both of them are colloidal suspensions. The carmine suspension is prepared by mixing 4 gm. of Grübler's carmine rubrum optimum in 100 cc. of a saturated solution of lithium carbonate, boiling, and filtering through filter paper. The ink suspension is a mixture of equal parts of commercial

Higgins' water-proof ink and distilled water. The carmine suspension is sterilized before use; the ink mixture need not be sterilized if sterile distilled water is used, as the ink contains enough camphor to keep it free from bacterial growth.

The fundamental principles involved are the supplying of two substances of different color, one through the air passages, the other through the circulation; that used in the latter instance must be one that will be taken up by the endothelial cells *in situ* and I know of only one such, a colloidal suspension of carbon, either in the ready made form just stated, or in that recommended by McJunkin. The latter is a 4 per cent suspension of finely triturated lampblack in normal salt solution, with 1 per cent of gelatin added to it. In this way the alveolar epithelium will be offered a red dye, the endothelium of the capillaries a black one, the origin of the cells that respond to the stimulus of the tubercle bacilli being readily traced by their color. The question of the specificity of the carbon for the vascular endothelium has been presented at length in the two preceding papers of this series, so any further discussion will be omitted here.

Histology of the Alveolar Epithelium.

Before proceeding to the consideration of the experiments done along these lines, it will be necessary to discuss the histology of the alveolar epithelium, for much error may result from misconceptions as to its structure. Once a definition of a typical alveolar epithelial cell has been accepted, the investigator will know what to look for when searching for such cells and will realize that not all are globular, with the bean-shaped nucleus and other characteristics of the endothelial leucocyte.

The epithelium of the alveoli, as well as that of the infundibula, is not a continuous layer of nucleated cells, but a membrane of varying thickness, composed of several types of cells, non-nucleated plates, and flanges, or processes, of nucleated cells. There are areas where one finds groups of nucleated cells very similar to those of the endothelium and practically indistinguishable from them. There are more or less cuboidal nucleated cells which extend almost through the alveolar wall and around which the capillaries twist, but whose alveolar

surface is more or less flared out to become continuous with adjacent cells. Thirdly, there are large, flat cells with broad plates extending from them, in which the nucleus is situated in a small mass of protoplasm more dense than the rest. These extending plates may represent similar cells that have lost their nuclei, or the processes, or flanges, of the flat, nucleated cells; both theories are advanced, the former being the most commonly encountered.

A cross-section of one of these plates is extremely difficult to see, as the cytoplasm has a refractive index that approaches that of Canada balsam very closely; but they can be made out under suitable conditions. It is possible to demonstrate them by injecting the fresh lung with a 0.25 per cent solution of silver nitrate, when the demarcation becomes evident. In my preparations they are best seen in tangential planes of section in material fixed in neutral formaldehyde; Helly's fluid shrinks them and causes fenestration of the cytoplasm. How they are formed from the cuboidal epithelium which precedes them in the fetus is still a matter of dispute. Bremer's work on the opossum lung would indicate that they are, in reality, flanges, or processes, of epithelial cells that are stretched out thin over capillaries. The reader is referred to such text-books as those by Ellenberger, Minot, Stöhr, etc. That most of these cells do not resemble the endothelial leucocyte can be seen at a glance; that they desquamate in the forms just described is readily demonstrable.

The group of roughly cuboidal cells is the most difficult to classify in regard to origin. Without ink injections the question seems unanswerable, as they look exactly alike; with ink in the cytoplasm of some of them, we can assume that these are endothelial. It would seem advisable to study the question of cell types in animals that are vitally stained, using a tridimensional wax reconstruction technique. That some of the cells in the groups which so closely resemble the endothelium may really be such, must be kept in mind. They might migrate from the capillary walls and collect in these situations, just as they do in the other tissues in the body, there to act as potential phagocytes in an organ where phagocytosis is very necessary. This is not a new idea; that many endothelial phagocytes are normally present in the lung is well recognized; their origin in the capillary walls and storage in the clumps of cells already described would, it seems, be perfectly natural.

Reaction to Pigments Alone.

Before describing the experiments in which tubercle bacilli were injected into the trachea, it would be well to know the reaction to pigments alone. In one rabbit ink was injected intravenously, and the animal died about an hour later while going under ether. Two rabbits were injected intravenously with 5 cc. of Higgins' ink and distilled water in equal parts and intratracheally, under an anesthetic, with 2 cc. of the lithium-carmine preparation. One was killed 24 hours later, the other 6 days later. The lungs and bronchial tree were removed *en bloc* and the former distended to three-quarters their normal expanded volume by injecting 4 per cent neutral formaldehyde. They were then sliced thin and fixed either in formaldehyde or Helly's fluid. They were stained with Delafield's hematoxylin, eosin-methylene blue, and Mallory's phosphotungstic acid hematoxylin.

In the animal in which the ink was used alone, the carbon is distributed in the endothelium of the interalveolar capillaries, and to a slight degree in the circulating mononuclear leucocytes, while some of it lies free in the circulating blood. It is also found in large cells in the alveoli, as described below. In the two other animals the ink distribution is the same, more appearing in the large cells in the alveolar spaces after 6 days than after 24 hours. There is a slight polymorphonuclear leucocytosis, with a scanty exudate of these cells after 24 hours; this is not seen in the 6 day preparations.

The large intraalveolar mononuclears resemble macrophages in every way; they are of irregularly globose, or obtusely angulated outline and have vesicular reniform nuclei and may contain vacuoles. They lie in the lymph spaces just below the epithelium, or free in the alveolar sac, or fitted into indentations in the alveolar surface of the epithelium, which surrounds them on three sides. Sometimes they seem to be interposed between two epithelial plates, as though penetrating the lining, and they can be seen, not infrequently, leaving the capillaries and lying athwart the lymphatics, with a portion of their cytoplasm penetrating the epithelium, in the act of migration. These cells are all important in the work at hand, for they play the principal part in the reaction to the tubercle bacillus in the air-borne infection and are evidently the cells which Sewell thought to be epithelial.

They show a few carbon granules as early as 1 hour after injection and take it up steadily for 6 or 7 days, when the experiments terminated. A few may be loaded with the ink particles, especially when in the alveolar wall; sometimes they are filled with carbon when free in the air space; usually, however, those that migrate contain less than those that do not. The foreign matter may hinder their motility to some extent. A little carbon is found in occasional polymorphonuclear leucocytes, but as a rule it is confined to the mononuclear cells. It is never found in the bronchial epithelium, or in any cells of clearly epithelial type in the alveoli. That it could be taken up by the epithelium from the blood stream seems highly improbable in the light of our present knowledge of the selectivity of the endothelium for this substance. The ink never seems to lie free in a position where the epithelial cells could phagocytose it, and the idea that it could be passed from the endothelial to the epithelial cells is not worth serious consideration.

The mononuclear migrating cells take up the carmine diffusely at first; after 5 hours, as seen in later series of sections, only one or two granules appear; after 24 hours the granular stain is complete and very beautiful. In the 6 day control animal these cells are found scattered in twos and threes in the alveolar sacs and bronchial lumina, or grouped in small masses in the alveoli and infundibula. They do not tend to form syncytia and show an intense granular carmine stain. One can also find them in the lymphatics and lymph nodes of the lung tissue and mediastinum. The epithelium of the bronchi and alveoli does not take the carmine except in a very faint, irregular, and diffuse manner. Thus the tissue reaction to the pigments alone is of a very slight and transitory character.

Experimental Pulmonary Tuberculosis.

In the actual experiment on the reaction to the tubercle bacilli the following procedure was carried out. Two stock strains of bovine tubercle bacilli were utilized and two methods were employed in preparing the suspensions used. In the first case one or two loops of dry culture on glycerol agar were rubbed up as finely as possible with a flat platinum needle and gradually suspended in 10 cc. of the lithium-carmine preparation used in the control tests, to be mechanically shaken for from ½ to 1 hour. The bacilli were first rubbed into a paste, more fluid was added, and the needle was bent into a J and rapidly spun between the

thumb and index finger until a fairly uniform cream was obtained. In the second instance a glass bulb pestle was used to triturate the same amount of culture into a thin film on the walls of a test-tube, fluid being added gradually and the whole shaken as before.

A series of rabbits was then injected intravenously with dilute Higgins' ink as in the controls; they were then anesthetized and 2 cc. of the suspended tubercle bacilli in lithium-carmine introduced into the trachea, just below the larynx, by means of a hypodermic syringe and needle. After a lapse of 1 hour, 5 hours, 1, 2, 3, 4, 5, 6, and 7 days the rabbits were killed by anesthetizing and injecting neutral 4 per cent formaldehyde into the beating heart. The lungs were then removed and treated as in the controls. The 5 and 24 hour lesions were produced in duplicate, one pair of rabbits receiving one strain, the other the second strain of tubercle bacilli.

The lesions resulting in all cases varied slightly with the individual and with the type of suspension used, the coarser type causing changes more like the usual tubercles and tuberculous bronchopneumonias, while the finer caused lesions that resembled caseous lobar pneumonia and were accompanied by fibrinous pleuritis (*cf.* Sewell's case).

The development of the experimental lesions, observed at the intervals indicated, proceeds as follows: First there is a widespread desquamation of the epithelium of the affected alveoli, often with destruction of their entire wall, including the capillary; large, flat cells, plates, and the debris thereof are found lying free in the air sacs and the lumina of the bronchi. Sometimes there is swelling of the epithelial plates, which peel off at their edges or gradually disintegrate. They may appear as a broad, hyaline, amorphous band at the periphery of the alveolus. The elastic fibers, as shown by Weigert's stain, also swell and undergo fragmentation as the process goes on. Many polymorphonuclears appear in the capillaries and migrate into the air vesicles, and with them comes an ever increasing number of large, ovoid, or slightly polygonal cells, which contain carbon and carmine at first to a slight, later to a marked degree (Fig. 1). They are in no way different from the mononuclear migratory cells described under the control experiment; at first they contain little or no ink, as they were presumably free in the alveoli before it was administered, but later they take it up in the vessels and appear in the alveoli with many granules in their cytoplasm, as can be seen in the photomicrographs.

As early as 5 hours after injection one can see marked proliferation of the endothelial cells of the capillary wall (better seen after 24 hours as in Fig. 2). Mitotic figures and ink globules are easily found. The endothelium is swollen and the cells are often in layers, or form nodal thickenings. Many figures of eight nuclei are present, and some swollen and distorted forms can be demonstrated, suggesting amitosis. The epithelium, on the other hand, does not show regenerative activity until the 3rd or 4th day, when it begins to proliferate, chiefly in the neighborhood of the bronchioles (Fig. 3). Here too, nodal heaps of cells occur, but they lie on the epithelial surface and project into the alveoli. Their cells are paler, more reticular, and contain no carbon, though they may take up a few bacilli. After 2 or 3 days the epithelium forms the familiar sheets of cuboidal cells on the alveolar wall and shows less activity. These sheets not infrequently grow over and enclose the masses of endothelial migratory cells which form in the alveolar spaces, sometimes almost entirely covering them with an epithelial envelope.

By the 4th day tubercles are found in the interstitial tissue, at the site of small collections of lymphoid cells (Fig. 4). They are composed of mononuclear cells containing ink, a few with the morphology of these but without carbon, and a few of the lymphocytes already present in the nodules, with a scattering of polymorphonuclears which have been attracted to the lesion. A day or so later the mononuclears in the alveolar spaces, which are now present in large numbers, fuse to form anastomosing masses of tubercles and syncytia, or giant cells (Fig. 5). In the series resulting from the injection of the coarser suspension of bacilli these areas are isolated and patchy (Fig. 6); in those in which the finer suspension was used the entire section is solid with the mass of mononuclear migrants, in many of which are mitotic figures, as in Fig. 3, showing that they are still actively growing. Numerous syncytia, commonly miscalled giant cells, are present and contain ink, carmine granules, and tubercle bacilli (Fig. 5). They first appear on the 3rd day after injection. The mononuclear endothelial cells of the exudate are often loaded with tubercle bacilli, as well as with ink and carmine (Fig. 6). While the intraalveolar tubercles have a predominatingly red color, that of the interstitial variety is black. On the 5th day these two types tend to fuse with one another,

including all the tissue between them, and large conglomerate areas result, which represent localized foci of tuberculous bronchopneumonia. As will be seen in Fig. 7, the fibroblasts that are included in these fusions begin to grow through the masses of endothelial cells and form interlacing bands of young connective tissue.

The reaction where a widespread lobar type of pneumonia resulted is essentially the same; there are more polymorphonuclears in the exudate and many erythrocytes are present. Both of these, with the endothelial mononuclears, fill whole sections of a lobe and, becoming caseous, form the exudate which gives this type of tuberculous pneumonia its name. The gelatinous exudate also appears and is found in alveoli where there is less active inflammation and cellular reaction and whose lymphatics are always widely dilated. Undoubtedly this is formed from coagulated lymph and a certain amount of very delicate fibrin, which can be demonstrated by using phosphotungstic acid hematoxylin either as a fine network or in a finely granular form, almost always without cellular admixture. The destruction of the elastic fibers is most marked in this type of inflammation; the fibrillæ are broken into very short, curly structures, not unlike large bacilli in their appearance. Here, then, the process is more diffuse and acute than in the cases in which the bacilli were less finely and evenly suspended and hence apt to enter the lung tissue in clumps.

The large phagocytic endothelial cells can be found in the lymphatics in this experiment, as they were in the controls. They are recognizable by their carmine and by the fact that tubercle bacilli can be demonstrated in their cytoplasm. That they contain ink in this case would, alone, prove that they came originally from the vascular endothelium; but the presence in these cells of the carmine and bacilli, which were introduced intratracheally, indicates that the cells come from the alveolar spaces. Hence we have pigmentary evidence that they have originated in the capillary walls, migrated to the air spaces, taken up bacilli and carmine, and, reentering the lymphatics, have carried these to the lymph nodes. Why they do so is not evident. This throws an interesting light on the spread of tuberculosis by means of migrating phagocytes; moreover, it furnishes additional evidence as to the endothelial origin of these cells, for such behavior is not characteristic of epithelium as we usually understand it.

The lymph nodes of the peribronchial lung tissue, as well as those of the mediastinal peribronchial group, show progressive increase in reticular cells and decrease in lymphocytes and lymphoblasts; small tubercles develop in the pulmonary nodes on about the 6th day. Most of these tubercles seem to be caused by an extension of the infection from tubercles in their immediate neighborhood. If bacilli carried to the nodes by phagocytes cause a development of tubercles in them, such a process would take place later, as there is little direct evidence thereof in this experiment, which represents a lapse of but 7 days after the introduction of the bacilli. Lymphocytes appear in the tubercles on about the 6th day; they seem to play no part in the primary formation of these lesions.

Another series of experiments is now in progress, a full report of which will appear in the near future. In these each rabbit receives in an ear vein 0.01 mg. of tubercle bacilli, of the same strain as that used in half the animals of the foregoing experiment. Niagara blue 3 B, an American form of trypan blue, is given intraperitoneally and Higgins' ink intravenously until the animals are vitally stained. The tubercles that result in the lung are of the miliary variety, almost all interstitial in location, apparently originating in, or near blood vessels and lymphoid tissue in the vicinity. They are deeply stained with ink and do not appear to take on much of the blue. There is little or no intraalveolar exudate or tubercle formation, in sharp contrast to the preceding experiment, in which the intratracheal infection resulted in striking exudation into the alveoli. In some places where the tubercles have become well developed and caseation is in an advanced stage, there is a spread to the alveoli (7 weeks), which gives rise to a reaction slighter than, but similar to that in the air-borne infection. This will be described in detail in the next paper, in connection with the lesions in the liver, spleen, kidney, etc., which, of necessity, result from a generalized hematogenous infection with tubercle bacilli. There is no doubt that there is a striking difference between the two types of infection when experimentally produced. All the hematogenous tubercles are composed of cells which contain more ink than do those found in the tubercles in the intratracheal infections. If the ink is administered for a while and the injections are then stopped, the tubercles are found to be deeply pigmented in

the older portions, while the newer zones of these lesions and the more recently formed lesions show very little carbon. For this reason it is necessary to continue the ink injections at least twice a week until the animal is killed.

Comparing the results of the experiments on the two types of infection, we find that the interstitially formed tubercles of the air-borne type compare well with those that predominate in the hematogenous type; they probably originate in the lymphatics in the first case and hence present this striking similarity. In the experiments on hematogenous infection we have no difficulty in tracing the origin of the epithelioid cell to the vascular endothelium; one can find tubercles produced as crescents in the lumina of medium sized capillaries, composed of cells that are so loaded with ink that their architecture is almost obscured. These tubercles can also be found in intratracheally infected series, but are more difficult of demonstration, for in this case we are dealing with secondary lesions, caused by bacteria which have escaped from the air vesicles, a condition which does not occur very frequently. The marked indifference of the epithelium to the hematogenous infection is another point against the theory that this tissue is the primary respondent to the tubercle bacillus.

It will be seen from what has been said that the findings in these experiments do not differ materially from those of Wechsberg, Watanabe, or Sewell; owing to the intravenous use of a colloidal suspension of India ink, however, the conclusions are decidedly different from those of the last two observers. If this material is not specific for the vascular endothelium, or at least selective enough in its action to be considered practically specific, this work must be discarded and new lines of approach devised before the question can be settled definitively. It appears that a specific agent has finally been found for marking the endothelial cell *in situ* and following it through its wanderings.

SUMMARY.

1. The injection of a colloidal suspension, or sol, of carbon into the veins of a living animal, as recommended by McJunkin, furnishes an apparently reliable means of tracing the so called epithelioid cell of the pulmonary tubercle from its origin in the vascular endothelium to the lesion.

2. Experimental tubercles are formed in the lung, as in the liver, primarily by cells originating in the capillary endothelium. These cells are probably present in small numbers in the normal lung, lying free both in the alveolar wall and the air vesicles. In response to infection they proliferate in the capillary walls in the vicinity of the invading organisms, migrate in steadily increasing numbers, and, arriving at the site of the infection, further multiply and to some extent fuse to form the syncytia known as giant cells.

3. The epithelial cell takes no active part in the process; its proliferation tends to repair denuded surfaces and is regenerative rather than combative or phagocytic in nature. This cell is free from carbon and stains only diffusely with carmine, in contradistinction to the endothelial cell which readily takes up both pigments in granular form.

4. The cells of endothelial origin not only phagocytose tubercle bacilli, but carry them into the tissues, for example into lymph nodes, by way of the lymphatics, or into other lung lobules by way of the air passages, in which they are readily demonstrable.

I am indebted to Professor J. Lewis Bremer for his kind permission to use the photomicrographic apparatus of the Department of Anatomy, and to his staff for their assistance and cooperation in its use.

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EXPLANATION OF PLATES.

PLATE 87.

FIG. 1. 5 hours after injection with tubercle bacilli. Note the large endothelial migratory cells in the alveolar spaces and the dilatation of the capillaries and lymphatics. × 1,000.[1]

FIG. 2. 24 hours after injection. The alveolar walls are thickened; polymorphonuclears are more numerous. × 1,000.

FIG. 3. 4 days after injection. Epithelial proliferation and partial fusion of the migrated endothelial cells, one of which shows a diaster. Carbon is not present in the epithelium, but is clearly seen in the intraalveolar mass of cells. × 1,000.

FIG. 4. 4 days after injection. The interstitial type of tubercle, very much richer in carbon than the intraalveolar form. × 1,000.

PLATE 88.

FIG. 5. 6 days after injection. Three well developed syncytia. Note the contained carbon particles. Several smaller cells with carbon are present. × 438.

FIG. 6. 1 week after injection. Part of an intraalveolar tubercle, showing carbon in the cells, as well as tubercle bacilli. × 657.

FIG. 7. 1 week after injection. Low power photomicrograph of an entire lesion, of the conglomerate, intraalveolar type. The streaks are groups of fibroblasts; organization is under way. × 163.

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[1] The figures of magnification are approximate but fairly accurate.

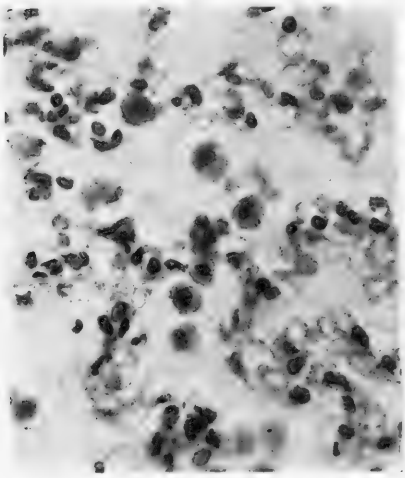

FIG. 1.

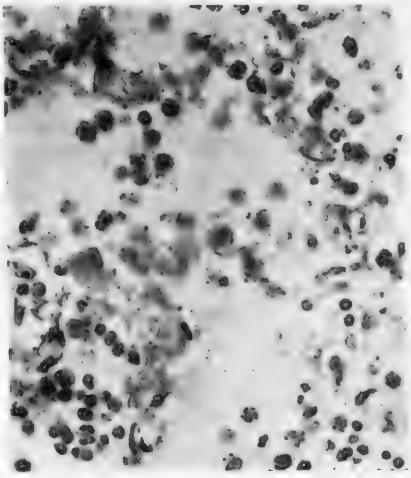

FIG. 2.

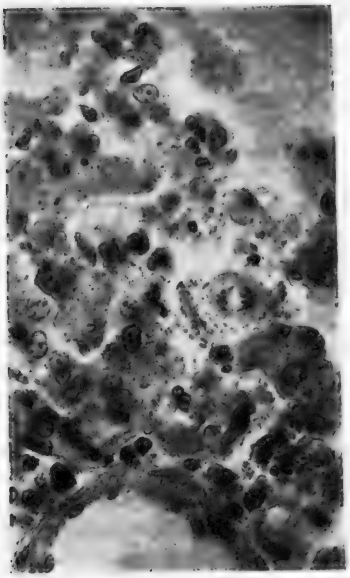

FIG. 3.

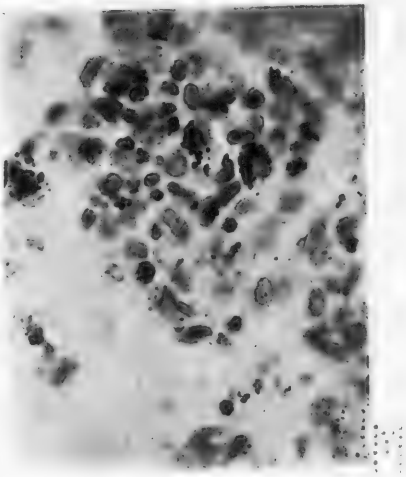

FIG. 4.

(Foot: Endothelial reactions. III.)

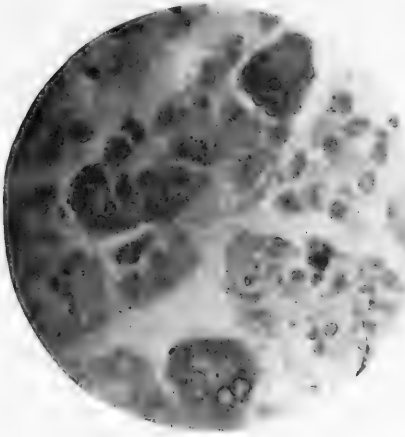

FIG. 5.

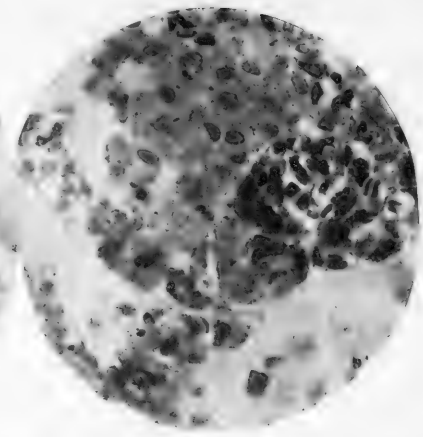

FIG. 6.

FIG. 7.

STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

I. Proteolytic Enzymes.

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Study of the biology of pneumococcus has led to a knowledge of certain biochemical characters which are common to the species as a whole, and to the recognition of fixed antigenic properties which serve to distinguish type differences within the species. The antigenic properties are inherent in the specificity of the bacterial protein and are detectable only by serologic reactions by means of which type relationships are recognized. The biochemical characters, on the other hand, are possessed in common by most pneumococci regardless of type differences, and are intimately associated with the life processes of the organism. These metabolic functions, upon which life of the cell depends, are in most instances referable to enzyme action. The presence or absence of a particular enzyme or group of enzymes determines largely the cellular activities of a microorganism. With the hope, therefore, of acquiring a better understanding of the way in which pneumococci adapt themselves to different environments, both in satisfying their nutritional needs and in exhibiting their invasive properties, the present study on the nature of the intracellular enzymes of this organism has been undertaken.

In the isolation and study of the enzymes of pneumococcus, apart from the living cell to which they are so intimately bound, use has been made of the fact that this organism undergoes rapid and complete solution in the presence of bile. Moreover, bile dissolves the bacterial cell with little or no change in the specific antigenic substance and with little or no injury to other demonstrable intracellular substances, such as the endohemotoxin. By dissolving the pneumococcus in bile and testing the cell-free solution on suitable substrates, enzymes are readily demonstrable. These enzymes have been found

to possess to a remarkable degree the power of actively hydrolyzing peptones to simpler peptides and amino-acids, of converting carbohydrates to simpler products, and of splitting esters to fatty acids. In demonstrating carbohydrate cleavage, however, bile was found to inhibit completely the hydrolysis of sucrose and starch, and another method of preparing the enzyme solution was necessary. This point will be emphasized in the paper on the intracellular invertase and amylase of pneumococcus (1).

The present paper concerns itself with the study of the proteolytic enzymes of pneumococcus. The intracellular nature of the enzymes, the influence of hydrogen ion concentration, the effect of age and concentration of the enzyme upon activity, and the relation of these enzymes to the virulence of the organism and to the mechanism of bile solubility will be discussed.

EXPERIMENTAL.

Bacteriological Methods.

Media.—The beef infusion broth containing 1 per cent peptone was prepared as previously described (2) except that (*a*) 2 gm. of dibasic phosphate per liter—anhydrous sodium phosphate or potassium phosphate—were used instead of 5 gm. per liter of sodium chloride, and (*b*) the medium was adjusted to a pH of 7.8.

Bile.—The ox bile used in preparing the pneumococcus enzyme solution was autoclaved, filtered, and again autoclaved as previously described (2).

Sterility Controls.—No antiseptics were used. The sterility of the enzyme solution was tested in broth and on blood agar plates. After addition of enzyme solution to the substrate, cultures of the mixtures were made by adding 0.1 cc. to 5 cc. of plain broth. In all the experiments recorded these controls remained sterile.

Chemical Methods.

Preparation of Substrate Solutions.—2 per cent solutions of peptone or protein were made in distilled water and the reaction was adjusted to the desired pH. The 2 per cent solution was then diluted with

an equal volume of 0.1 M phosphate solution of the desired pH. The final concentrations, unless otherwise stated, were therefore 1 per cent of substance in 0.05 M phosphate solution. The phosphate solutions were prepared from Merck's special reagents (Na_2HPO_4 and KH_2PO_4) according to Sörensen's (3) tables.

Sterilization.—Unless otherwise stated, sterilization was accomplished by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The pH values were usually determined colorimetrically, with the series of indicators outlined by Clark and Lubs (4). The solutions were diluted with two volumes of redistilled water, and the indicators used in such strength that one drop was required per 3 cc. of diluted solution. The readings were made by the comparator method (Walpole). Sörensen's standard phosphate and Walpole's (5) standard acetate solutions were used. These determinations were frequently checked by the electrometric method.

Nitrogen Determinations.—Total nitrogen determinations were made by the Kjeldahl method. Amino nitrogen was determined by Van Slyke's (6) nitrous acid method. With the peptone solution the determinations were made directly on 2 cc. samples, by means of the micro apparatus. Determinations of the amino nitrogen of the protein solutions were done by one of two methods: (*a*) 10 cc. samples were deaminized for 15 minutes in the large Van Slyke apparatus, and the nitrogen liberated was read in the micro burette calibrated to 0.002 cc.; and (*b*) the protein was precipitated with colloidal iron in the manner described by Van Slyke, Vinograd-Villchur, and Losee (7).

In determining the peptide nitrogen of the peptone solutions the peptides were split to amino-acids by acid hydrolysis (Van Slyke (8)). The peptide nitrogen was then calculated as the increase in amino nitrogen.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Fairchild).

Experiment 1. (*a*) *Preparation of Enzyme.*—The washed bacterial residue from 2 liters of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was taken up in 15 cc. of 33 per cent dilution of sterile bile (bile, 5 cc., + water, 10 cc.) and placed in the ice box over night. A portion of this bile solution of pneumococcus was inactivated by heat, as a control.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution of various hydrogen ion concentrations were sterilized by the Arnold method on 3 successive days.

(*c*) *Sterility Control.*—No antiseptics were used. After the addition of enzyme solution to substrate, cultures of all digestive mixtures were made by adding 0.1 cc. of each to 5 cc. of plain broth. All cultures, including that of the bile solution of pneumococcus, were sterile.

The experiment was carried out as follows: Duplicate 20 cc. portions of the peptone substrate at reactions of pH 4.2, 5.3, 6.2, 7.0, and 7.6 were prepared. To one tube of each set, 1 cc. of enzyme solution was added, to the other 1 cc. of the inactivated enzyme solution. The tubes were then kept at 37°C. for 2 days. No antiseptic was used, sterility being maintained throughout by bacteriological technique. All cultural controls remained sterile, and after 48 hours the tubes were removed from the incubator for analysis. Duplicate amino-acid nitrogen determinations were made on 2 cc. samples by the Van Slyke method. The estimations of hydrogen ion concentration were determined colorimetrically on 5 cc. portions. The results are tabulated in Table I.

TABLE I.

Determination of Peptone-Splitting Activity (Fairchild's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.2	4.2	27.3	27.2	
2	5.3	5.3	27.5	32.3	4.7
3	6.2	6.2	27.6	45.3	17.7
4	7.0	7.1	29.5	49.8	20.3
5	7.6	7.6	28.2	47.9	19.6
Peptone.			28.2		

Distribution of Nitrogen per 100 Cc. of Substrate.

		mg.
Total nitrogen		107
Amino "	before hydrolysis	27.5
" "	after "	78.6
Peptide "	therefore equals	51.1

Analysis of Experiment.

The increase of 20.3 mg. of amino nitrogen per 100 cc. of substrate shows that 40 per cent of peptide nitrogen was split to free amino nitrogen.

In order to determine the proportion of the peptide that was digested, a nitrogen partition was done on the peptone solution. Total nitrogen on 2 cc. portions was determined by the Kjeldahl method. Peptide nitrogen[1] was computed as the difference between total amino-acid nitrogen before and after hydrolysis.

Action of Intracellular Enzymes of Pneumococcus on Peptone (Witte).

Experiment 2. (*a*) *Preparation of Enzyme.*—This was the same as in Experiment 1, except that the bacterial residue from 2 liters of Pneumococcus Type II (No. F 208) was dissolved in 15 cc. of 25 per cent solution of sterile bile in water.

(*b*) *Preparation of Substrate.*—The substrate was prepared as in Experiment 1, except that Witte's peptone was used instead of Fairchild's preparation.

(*c*) *Sterility Control.*—No antiseptic was used. All tubes including cultural control of the enzyme solution were proved sterile by subcultures as in the preceding experiment.

In Experiment 2 the peptone-splitting action of pneumococcus enzyme was tested on Witte's peptone. The experimental technique was the same as that described in the preceding protocol, except for the substitution of 1 per cent Witte's peptone for the Fairchild preparation. The Witte peptone substrate was analyzed for total nitrogen, and peptide nitrogen in the same manner. The results are given in Table II.

It is evident from Experiments 1 and 2 that pneumococcus contains within its cell an enzyme or enzymes capable of hydrolyzing peptides into amino-acids or simpler peptides. From 26 to 40 per cent of the peptide nitrogen present in the peptone substrate was split to amino nitrogen. The term "peptone" solution is used to indicate the mixtures of partially hydrolyzed protein products which are known commercially as "peptones." The considerable data available as to the chemical nature of these peptones show them to be mixtures of protein products of varying degrees of complexity. The amount of pep-

[1] By peptide nitrogen is meant nitrogen found in the peptide linkings, the $-CO-NH-$ groups that link the different amino-acids together in peptides, proteins, or intermediate products. The process of hydrolysis consists in the splitting of these peptide groups, from each of which is generated a carboxyl group and an amino group. Thus

$$RNH-CO-R^1+H_2O=RNH_2+R^1COOH$$

For further discussion of this point see Van Slyke, D. D., *Arch. Int. Med.*, 1917, xix, 56.

tide hydrolyzed was greater in the experiment in which Fairchild's peptone was used. The proportion of preformed amino nitrogen to total nitrogen in this preparation was greater than in the sample of Witte's peptone, indicating that, on the average, Fairchild's peptone consists of simpler intermediate protein digestion products than Witte's. This may be the reason that further digestion by the enzyme proceeded more rapidly in the Fairchild product, the enzyme attacking the simpler peptides of the preparation the more readily.

TABLE II.

Determination of Peptone-Splitting Activity (Witte's Peptone).

Tube No.	Final hydrogen ion concentration.		Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Inactive.	Active.	Increase.
	pH	*pH*	*mg.*	*mg.*	*mg.*
1	4.4	4.4	15.5	15.6	
2	5.0	5.0	15.6	21.2	5.6
3	6.0	6.0	15.4	32.4	17.0
4	7.0	7.0	15.5	39.6	24.1
5	7.8	7.8	15.5	39.4	23.9

Distribution of Nitrogen per 100 Cc. of Substrate.

		mg.
Total nitrogen		148
Amino "	before hydrolysis	15.5
" "	after "	108
Peptide "		92.5

Analysis of Experiment.

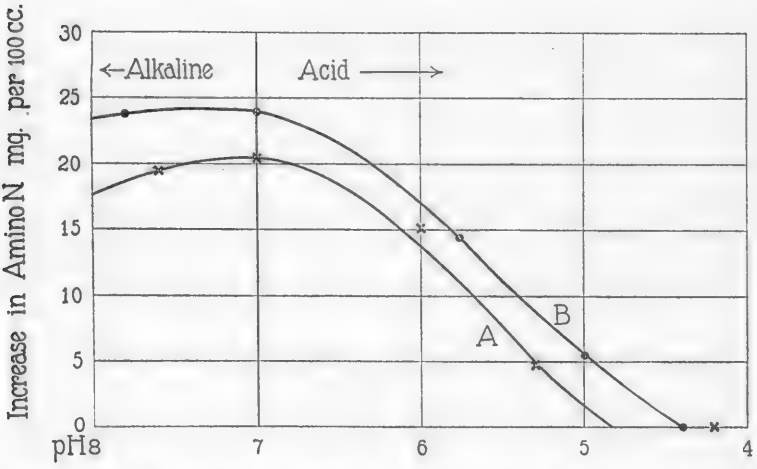
The increase of 24.1 mg. per 100 cc. of substrate shows that 26 per cent of peptide nitrogen was split to free amino nitrogen.

Effect of Hydrogen Ion Concentration on the Activity of Pneumococcus Peptonase.

Experiments 1 and 2 were planned to determine the relation of enzyme activity to hydrogen ion concentration. This relation is evident from the curves of Text-fig. 1, in which *A* represents Experiment 1 and *B* Experiment 2. The optimum activity of the peptone-splitting enzyme is from pH 7 to pH 7.8. With increase in acidity the

activity of the enzyme is increasingly retarded until complete inhibition results at pH 4.5.

These facts, that about 30 to 40 per cent of the peptide is hydrolyzed and that the optimum zone for activity of the enzyme is between pH 7 and 7.8, place it in the erepsin class of enzymes. Moreover, the fact that the curve of acid inhibition proceeds in a straight line to complete inhibition at a pH of about 4.5 indicates that the enzyme preparation used contains no pepsin and is, therefore, not a complex



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of pneumococcus peptonase. Curve *A*, Experiment 1; Curve *B*, Experiment 2.

of erepsin- and pepsin-like enzymes. Since it seemed desirable to maintain an open mind on the question as to whether the enzymotic action in these experiments is more closely allied to trypsin or erepsin, and in view of its relative rate of action on native protein, the term "peptonase" has been used throughout the remainder of this report. It is significant that the optimum reaction zone for the intracellular peptolytic enzyme corresponds with the optimum for growth of pneumococcus (9).

Use of Sodium Choleate in Demonstrating the Intracellular Enzymes of Pneumococcus.

It is well known that pneumococci undergo solution in the presence of bile salts as completely as in the presence of bile itself. In order to determine whether the substitution of sodium choleate for bile in dissolving the organisms exerted any influence on the activity of the intracellular enzymes the following experiment was carried out.

Experiment 3. (*a*) *Preparation of Enzyme.*—Pneumococcus Type I (No. G_2) was grown in 2 liters of plain broth for 18 hours at 37°C. The bacteria were removed by centrifugation, washed once in sterile isotonic salt solution, and suspended in 10 cc. of 5 per cent solution of sodium choleate. After 5 hours in a water bath at 37°C., the resultant solution of pneumococci was diluted with an equal volume of sterile distilled water. One portion of this solution was inactivated by heat and both were tested for activity in a peptone substrate.

(*b*) *Preparation of Substrate.*—20 cc. portions of 1 per cent Fairchild's peptone in 0.05 M phosphate solution were adjusted to the various hydrogen ion concentrations and sterilized by the Arnold method on 3 successive days.

This experiment was conducted in exactly the same manner as Experiment 1.

TABLE III.

Determination of Peptone-Splitting Activity of a Solution of Pneumococcus Obtained by the Use of Sodium Choleate.

Tube No.	pH	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
		mg.	*mg.*	*mg.*
1	5.0	37.9	42.9	5.0
2	5.4	37.9	50.7	12.8
3	5.8	38.7	53.4	14.7
4	6.2	40.4	55.2	14.8
5	6.6	41.6	59.2	17.6
6	7.0	42.2	60.3	18.1
7	7.4	39.6	58.9	19.3
8	7.8	40.7	58.3	17.6

From the data presented in Table III it is evident that when pneumococci are dissolved by sodium choleate there is liberated from the cell a peptone-splitting enzyme in the same manner as when solution of the organism is effected by the action of bile. It has also been

found that enzyme solutions obtained by disintegration of pneumococcus cells without the presence of bile or bile salts, by methods described in a succeeding paper (1), exhibit comparable activity. Therefore, bile salts are not essential to the action of the enzyme.

Effect of Age on the Activity of the Intracellular Peptonase of Pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme.*—The technique of preparing the enzyme solution in this experiment was similar to that described in preceding protocols, except that the bacterial residue from 2 liters of broth culture of Pneumococcus Type II (No. F 208) was dissolved directly in 10 cc. of undiluted bile. After 2 hours in the water bath at 37°C. and 1 hour at room temperature, the enzyme solution was stored in the ice box, and at intervals up to 43 days portions were removed and tested for activity.

(*b*) *Preparation of Substrate.*—1 per cent of Fairchild's peptone in $\frac{M}{30}$ phosphate solution of pH 7 was prepared and sterilized in the autoclave at 15 pounds pressure for 20 minutes.

(*c*) *Sterility Control.*—No antiseptics were used. Cultures of the original enzyme solution and each digestion mixture at the time of carrying out the several tests proved sterile.

The results are presented in Table IV.

TABLE IV.

Age Stability of Endopeptonase.

Enzyme solution kept at about 4°C. 1 cc. added to 20 cc. of peptone (Fairchild), pH 7. 24 hours at 37°C.

Age.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
days	*mg.*	*mg.*	*mg.*
0	37.4	58.8	21.4
1	37.4	59.7	22.3
2	40.8	62.6	21.8
6	37.4	53.6	16.2
20	40.8	54.4	13.6
43	23.4	32.1	8.7

The length of time an enzyme may remain active is dependent upon the conditions of its preservation. The age stability of an

enzyme in dried form is greater than that of the same enzyme in solution. In the present instance, the intracellular peptonase of pneumococcus dissolved in undiluted ox bile retained about 40 per cent of its activity for over 6 weeks.

Relation of Virulence of Pneumococcus to Enzyme Activity.

Experiment 5. (*a*) *Preparation of Enzyme.*—Plain broth cultures (1,500 cc.) of a virulent and avirulent strain of Pneumococcus Type II (No. F 208) were centrifuged; the bacterial sediment was washed once in sterile isotonic salt solution, then dissolved in 15 cc. of undiluted bile, and held in the ice box over night. Portions of the enzyme solutions were inactivated by heating in the autoclave at 15 pounds pressure for 20 minutes.

TABLE V.

Influence of Virulence of Pneumococcus upon the Activity of the Intracellular Peptonase.

2 cc. of enzyme solution added to 20 cc. of peptone solution, pH 7. Incubated at 37°C. for 24 hours.

Pneumococcus Type II.	Minimum fatal dose.	Amino nitrogen per 100 cc. of substrate.		
		Inactive.	Active.	Increase.
	cc.	*mg.*	*mg.*	*mg.*
No. F 208 A	0.000001	39.8	68.6	28.8
" F 208 "B"	Greater than 1	39.2	67.8	28.6

(*b*) *Preparation of Substrate.*—1 per cent peptone (Fairchild) in $\frac{M}{30}$ phosphate solution, pH 7, was sterilized in the autoclave.

(*c*) *Sterility Control.*—No antiseptics were used. Each tube in the experiment was tested for sterility by subculture and yielded no growth.

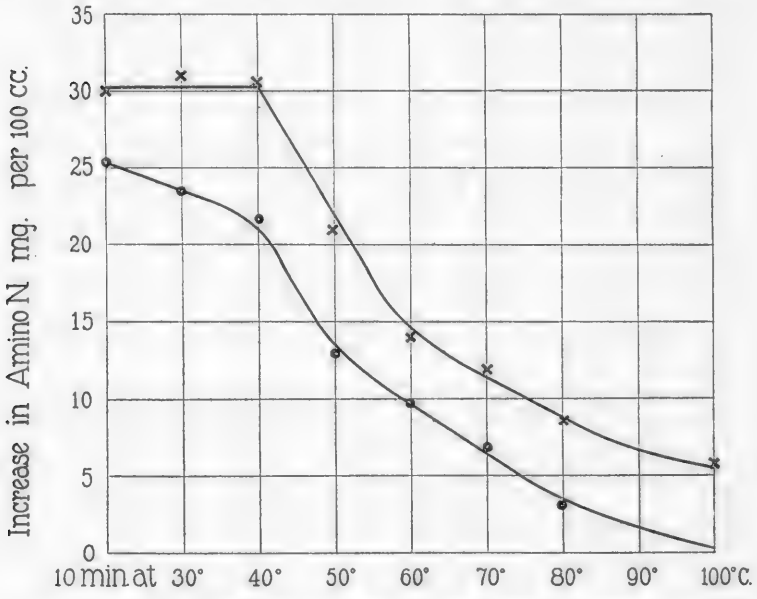
The development of a technique for the demonstration of endoenzymes made it possible to submit to experimental proof the question whether differences in virulence are in any way related to the activity of the intracellular enzymes. For this purpose a strain of Pneumococcus Type II (No. F 208) was chosen. This organism was originally isolated from the blood of a patient suffering from lobar pneumonia. The virulence of the strain, maintained by animal passage, was such that 0.000001 cc. of broth culture injected intraperitoneally into white mice proved fatal in 24 to 48 hours. A subculture of the same strain, the virulence of which had been attenuated by cultural methods, failed to kill mice in doses of 1 cc. Enzyme solutions from comparable amounts of bacteria were prepared from the virulent and avirulent cultures of this strain. The respective

enzyme preparations were tested for peptonase action by adding 2 cc. of each to substrates of 20 cc. of 1 per cent peptone solution adjusted by phosphates to pH 7. After 24 hours at 37°C. the degree of enzyme action was determined by measuring the increase of amino nitrogen as indicated in Table V.

From Table V it appears that loss of virulence is not associated with a corresponding loss of enzymotic activity. Under the conditions of this experiment at least, the amount of hydrolysis of peptone by the endoenzymes of the avirulent strain was equivalent to that of the virulent organism.

Effect of Heat on the Intracellular Peptonase of Pneumococcus.

Sensitiveness to heat is a biologic character of all enzymes. In determining the influence of heat upon dissolved enzymes, the degree of temperature, the length of exposure, and the reaction of the solu-



TEXT-FIG. 2. Heat stability of intracellular peptonase of pneumococcus. The lower curve represents the results after 24 hours incubation at 37°C., the upper curve after 48 hours at 37°C.

tion are closely interrelated. The optimum reaction for activity of the endopeptonase of pneumococcus, pH 7.4, and an exposure of 10 minutes were arbitrarily chosen, and the temperature alone was varied as shown in Text-fig. 2.

Experiment 6. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of plain broth culture of Pneumococcus Type II (No. F 208) was dissolved in 20 cc. of sterile ox bile and held in the ice box over night.

(*b*) *Preparation of Substrate.*—1 per cent peptone solution (Fairchild) in 0.05 M phosphate solution adjusted to pH 7.4 was sterilized in the autoclave.

(*c*) *Sterility Control.*—Sterility was proved by subculture from each tube.

1 cc. of the enzyme solution with a pH of about 7.4 was placed in each of eight sterile tubes, and these in turn were immersed in water baths at 30°, 40°, 50°, 60°, 70°, 80°, 90°, and 100°C., respectively, for exactly 10 minutes. On removal the tubes were immediately cooled, and to each were added 10 cc. of the sterile substrate. After 24 and 48 hours incubation at 37°C. samples were removed for analysis. The results are plotted in Text-fig. 2. The heat sensitiveness of the enzyme manifests itself in a progressive loss of activity after exposure for 10 minutes to increasing temperatures, until at 100°C. little or no activity remains.

Effect of Concentration of Enzyme on the Activity of the Intracellular Peptonase of Pneumococcus.

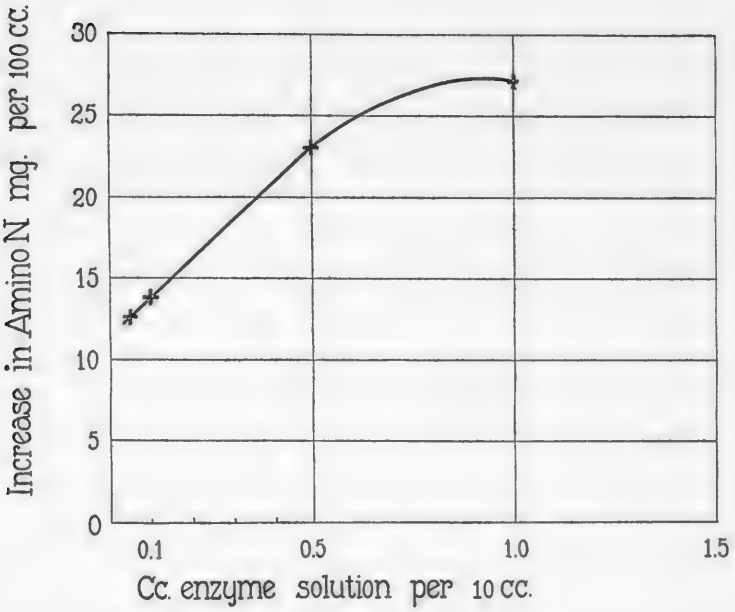
Experiment 7.—The same preparation of enzyme and substrate used in Experiment 6 was employed in this test, a bile solution of Pneumococcus Type II and a 1 per cent solution of peptone (Fairchild's) in 0.05 M phosphate mixture, pH 7.4.

The dissolved enzyme was diluted with bile so that 1 cc. of the final solution added to 10 cc. of peptone substrate contained 0.05, 0.1, 0.5, and 1 cc. respectively of the original enzyme solution. The results are plotted in Text-fig. 3.

For a more exact study of the dynamics of this enzyme it would be desirable to repeat this experiment with shorter digestion periods and smaller amounts of enzyme. It is evident, however, that under given conditions the rate of hydrolysis is proportional to the concentration of enzyme.

Occurrence of Enzymes in Culture Filtrates of Pneumococcus.

The experiments thus far have dealt entirely with intracellular enzymes. It was considered probable that the culture medium itself



TEXT-FIG. 3. Effect of concentration of enzyme on the activity of the intracellular peptonase of pneumococcus.

would contain similar enzymes which had either diffused out from the cell during growth or had been liberated by disintegration of the organisms in the culture fluid. In order to determine the validity of this assumption, the following experiment was undertaken.

Experiment 8. (*a*) *Preparation of Filtrate.*—100 cc. of an 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was filtered through a Berkefeld candle N. The filtrate, pH 7, was tested for sterility by adding 5 cc. to 100 cc. of fresh broth and incubating at 37°C. In testing the sterility of culture filtrates of pneumococcus it is not sufficient merely to incubate the filtrate itself, but it is essential to inoculate fresh broth. This is probably due to the fact, recorded in a previous paper (Avery and Cullen (10)), that further growth cannot be initiated in the filtrates of a plain broth culture of pneumococcus even though the reaction is readjusted to the optimum hydrogen ion concentration. A portion of the enzyme-containing filtrate was inactivated by heat, to serve as a control.

(*b*) *Preparation of Substrates.*—These consisted of the sterile culture filtrate and a 1 per cent solution of peptone (Fairchild) in phosphate mixture adjusted

to pH 7, the same hydrogen ion concentration as the filtrate. The peptone solution was sterilized in the autoclave.

In determining the presence of enzyme in culture filtrates of pneumococcus the amount of cleavage was determined, (*a*) as the result of further action of the enzyme on the peptones present in the broth filtrates and (*b*) as the result of this action plus the action on additional peptones. This was accomplished by the procedure indicated in Table VI, three tubes of substrate being used, in all of which the final volume was 20 cc. The first tube contained 10 cc. of filtrate and 10 cc. of sterile water, the second, 10 cc. of filtrate and 10 cc. of 1 per cent peptone solution, and the third (the control), 10 cc. of 1 per cent peptone solution plus 10 cc. of sterile water. The determinations of amino nitrogen in the digestion mixtures before and after incubation are given in Table VI.

TABLE VI.

Presence of Peptonase in Culture Filtrate of Pneumococcus.

Tube No.	Sterile filtrate unheated; pH 7.	1 per cent peptone solution; pH 7.	Water.	Amino nitrogen per 100 cc. of final solution.		
				Before digestion.	After digestion.	Increase.
	cc.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*
1	10		10	54.9	73.4	18.5
2	10		10	54.7	73.0	18.3
3		10	10	24.0	24.0	
4		10	10	24.0	24.0	
5	10	10		78.9	106.8	27.9
6	10	10		79.0	106.8	27.8

Analysis of Table VI.

Increase in amino nitrogen in filtrate alone..... 18.4 mg. per 100 cc.
" " " " " " + peptone.... 27.9 " " 100 "
" " " " due to action of enzyme
on added peptone..... 9.4 " " 100 "

From Table VI it is clear that under the conditions of the experiment the bacteria-free filtrate contained an active enzyme which continued to hydrolyze the excess of available peptide in the medium and in addition attacked the added peptone.

The demonstration of the presence of an enzyme in culture filtrates of pneumococcus after 18 hours incubation does not necessarily imply that the enzyme is a true secretory product which is elaborated during

growth and given off into the medium in the manner of exotoxins. Under optimum cultural conditions pneumococcus reaches its maximum growth relatively early (Chesney (11)), after which involution and disintegration of the bacterial cells soon begin. During this later period, the intracellular hemolysin of pneumococcus, which cannot be detected free in the medium during the early phase of active growth, is also released from the disrupted cell and can be demonstrated in the culture fluid. If the peptonase is an endoenzyme, its absence in filtrates during the period of active growth should be demonstrable.

In order, therefore, to test for presence of enzyme in the culture fluid before cell death and disruption with consequent liberation of intracellular enzyme occurred, the following experiment was carried out.

200 cc. of plain broth, pH 7.8, were inoculated with 0.5 cc. of a 5 hour culture of Pneumococcus Type II (No. F 208). After 5 hours incubation marked growth was apparent and the acidity had increased to pH 7.5. The culture was then centrifuged and the supernatant fluid was filtered through a tested Berkefeld candle N. This filtrate was kept in the refrigerator until its sterility was proved by culture. The sterile filtrate was then tested for enzyme activity as in the preceding experiment.

It was found that during the phase of active growth the culture fluid freed from bacteria possessed no peptolytic activity. Moreover, in this same culture fluid the intracellular hemolysin, known to be liberated by cell disintegration, was likewise not demonstrable, but the soluble substance shown by Dochez and Avery (12) to be elaborated during the earliest phase of cell multiplication was detectable in considerable concentration. The occurrence, therefore, of peptolytic activity in autolyzing broth cultures and its absence in the culture fluid during the early phases of active growth make it evident that the peptonase is a true endoenzyme.

Effect of Exposure to Acid Reaction on the Intracellular Peptonase of Pneumococcus.

It has been observed that cultures of pneumococci grown in sugar-containing medium reach a final hydrogen ion concentration of about pH 5 to 5.2. At this point the organisms quickly succumb, the acidity

produced by their own metabolic processes being sufficient in itself to stop growth. Lord and Nye (13) have found that pneumococci will not live for more than a few hours in a solution the acidity of which is greater than pH 5.1. Experiments in this laboratory have shown that pneumococci subjected to an acidity of pH 4.5 to 5 for 2 hours are no longer soluble in bile. Even when these organisms are removed by centrifugation, washed, and resuspended in phosphate solution at a pH 7.8, they remain insoluble in bile. The question arose whether this reaction of pH 4.5 to 5, which corresponds to the acid death-point of the bacterial cells, is also fatal to the intracellular enzymes, and whether this fact is of any significance in the phenomenon of bile insolubility.

To determine whether acid treatment injured the endoenzymes of pneumococcus the following experiment was carried out.

Experiment 9.—1 cc. of the enzyme solution used in Experiment 6 was treated with 4 cc. of 0.1 M potassium acid phosphate solution of pH 4.5. The reaction of the resulting solution was pH 5. After 2 hours at 37°C. the solution was readjusted to pH 7.4 by the addition of 0.3 cc. of N sodium hydroxide (calculated and verified on a separate sample), and 5 cc. of a 2 per cent peptone solution of pH 7.4 were added. As a control on activity, the untreated active enzyme was added to the same peptone substrate in similar proportions. The results are given in Table VII.

TABLE VII.

Effect on Peptonase of Exposure to Acid Reaction.

Final concentration of enzyme was 1 cc. per 10 cc. of 1 per cent peptone in 0.1 M phosphate solution of pH 7.4. 42 hours at 37°C.

Treatment of enzyme.	Amino nitrogen per 100 cc. of substrate.		
	Inactive.	Active.	Increase.
	mg.	*mg.*	*mg.*
Enzyme solution previously kept at pH 7.8.....	31.2	58	26.8
" " " adjusted to pH 5.0 and after 2 hrs. at 37°C. readjusted to pH 7.4.....	31.2	58	26.8

The intracellular enzyme in solution suffered no loss of potency after being subjected for 2 hours to an acidity of pH 5, for upon readjustment to the optimum hydrogen ion concentration of pH 7.4, the

acid-treated enzyme exhibited an activity comparable to that of the untreated enzyme. The endopeptonase of pneumococcus is evidently little influenced by this reaction change. The bile insolubility of pneumococci at the acid death-point is, therefore, not associated with destruction of this enzyme, but is probably referable to coagulative changes in the cell protoplasm.

Action of the Endoenzymes of Pneumococcus on the Proteins, Casein, Gelatin, Albumin, and Fibrin.

The data presented in the preceding protocols establish the fact that there is present within the bacterial cell an enzyme, or enzymes, capable of hydrolyzing peptones into amino-acids, and that this enzyme-complex manifests its optimum activity in a slightly alkaline medium. Simultaneous experiments were carried out with the same enzyme preparations to determine their action on the proteins, casein, gelatin, fibrin, and albumin.

Preparation of Enzyme.—In these experiments the enzyme solutions were prepared in the manner described in Experiment 6.

Preparation of Substrates.—2 per cent gelatin, albumin (egg), and casein solutions were adjusted to pH 7.4 and diluted with an equal volume of 0.1 M phosphate solution of the same hydrogen ion concentration and then sterilized in the autoclave. The fibrin substrate D was prepared by adding 0.2 gm. of dried commercial fibrin to 10 cc. of 0.05 M phosphate solution of pH 7.4 and was sterilized in the autoclave. The fibrin substrate F was freshly prepared from 10 cc. portions of sterile oxalated rabbit plasma. Sterile calcium chloride was added and the fibrin clot was washed in sterile salt solution and transferred to 10 cc. of sterile 0.05 M phosphate solution of pH 7.4.

Experiment 10.—To 10 cc. portions of the sterile protein solution 1 cc. of enzyme solution was added, and the mixture placed at 37°C. for 5 to 7 days. The degree of digestion was determined as in the peptone experiments by amino nitrogen determination with one of the following procedures: (*a*) 10 cc. portions of the digestion mixtures were placed in the deaminizing bulb of the original large type of Van Slyke apparatus. 15 minutes were allowed for deamination and the nitrogen evolved was measured in a micro burette graduated to 0.002 cc. (*b*) After removal of the proteins by colloidal iron, amino nitrogen determinations were made in the manner described in preceding experiments. The results are presented in Table VIII.

Experiment 11.—In following the extent of digestion of the peptone solution control amino nitrogen determinations on active enzyme alone had shown that

TABLE VIII.

Proteolytic Action of Endoenzymes of Pneumococcus.

Protein.	Duration of digestion.	Method of analysis.	Amino nitrogen per 100 cc. of substrate.		
			Inactive.	Active.	Increase
	days		*mg.*	*mg.*	*mg.*
Casein.	5	(*b*)	1.9	6.3	4.4
"	12	(*b*)	1.3	3.4	2.1
Gelatin.	7	(*a*)	6.3	6.7	0.4
	12	(*b*)	2.8	3.5	0.7
Fibrin D.	5	(*a*)	2.5	4.0	1.5
" F.	31	(*b*)	4.2	7.1	2.9

the increase in amino nitrogen due to autolysis of the bacterial proteins was negligible. However, because of the small concentration of free amino nitrogen in the protein experiment more rigid control of this protein digestion was carried out. Duplicate 10 cc. portions of casein, albumin, and 0.05 M phosphate, all at pH 7.4, were prepared. To one tube of each pair 2 cc. of active enzyme were added, and to the other 2 cc. of heated enzyme. Tubes were incubated at 37°C. for 5 days. Each dilution was then diluted to 25 cc., 5 cc. portions were removed for total nitrogen determinations, and the remaining 20 cc. were freed from protein by precipitation with colloidal iron. The non-protein nitrogen of the filtrate and washings was determined by the Kjeldahl method. The results are presented in Table IX.

TABLE IX.

Proteolytic Action of Endoenzymes of Pneumococcus.

Proteolysis measured by increase in non-protein nitrogen. Digestion for 5 days at 37°C.

Solution.	Total nitrogen per 100 cc. of solution.	Non-protein nitrogen per 100 cc. of solution.			Protein digested.
		Inactive.	Active.	Increase.	
	mg.	*mg.*	*mg.*	*mg.*	*per cent*
Enzyme	25.7	14.8	16.8	2.0	20.0
Casein*	14.6	7.0	19.4	12.4	8.9
Albumin*	12.3	6.4	6.5		

* Protein figures corrected for nitrogen of enzyme solution added.

From Table IX it is evident that autolysis of the enzyme solution itself is occurring. This increase would, for the concentrations used in Table VIII, amount to about 0.5 mg. of amino nitrogen per 100 cc.; that is, the increase in the case of the gelatin experiment is due to the enzyme solution. The increase of 3 and 4 mg. in the case of casein and fibrin indicates a definite proteolysis.

The endoenzymes of pneumococcus are apparently able partially to hydrolyze the proteins, casein and fibrin, but not albumin or gelatin. This enzyme action on intact protein is distinctly less, however, than that which occurs in the presence of peptones.

DISCUSSION.

To present a critical review of the literature on the nature and action of bacterial enzymes would entail a task beyond the scope of this paper. Although extensive study of the enzymes of a large variety of different species of microorganisms has been made, comparatively little work has been done on the occurrence and character of the endoenzymes of pneumococcus.

Rosenow (14) demonstrated in extracts of virulent pneumococci and filtrates of broth cultures a proteolytic enzyme capable of hydrolyzing the proteins in meat broth, in ascites meat broth, and to a less extent the proteins of heated serum. He prepared extracts of pneumococci by suspending the bacteria from broth cultures in salt solution, adding ether, and allowing autolysis to proceed at 37°C. for 48 hours. The bacteria were removed by centrifugation or passage through a Berkefeld filter. Rosenow found that the degree of disintegration of the bacterial cells was directly proportional to the amount of proteolysis as measured by formol titration. He has shown further that the toxicity of broth, culture filtrates, and extracts of pneumococci is associated with proteolysis due to the dissolved enzymes.

Evidence is presented in this paper of the existence of proteolytic enzymes within the cell body of pneumococcus. This enzyme or group of enzymes can be isolated from the living cell by dissolving the organisms in bile or allowing them to cytolyze in phosphate solutions of pH 6.2. In the latter method alternate thawing and freezing of the bacterial suspension greatly facilitate the extraction process by disrupting the bacterial cells.[2] By these methods the intracellular

[2] See Avery and Cullen (1).

substances pass into solution in a medium in which they retain their activity for a considerable period. The proteolytic activity of these enzymes is manifest in their ability to hydrolyze, to some extent, intact protein and to split to a striking degree intermediate products, such as peptones, into simpler peptides and amino-acids. It has not been determined whether the two processes, proteolysis and peptolysis, are functions of the same enzyme or the result of the action of two separate enzymes.

It is evident that the action on the intact proteins, fibrin and casein, is distinctly less than on simpler substances such as occurs in peptone mixtures. For this reason the larger number of experiments has been carried out with a partially hydrolyzed protein, commonly known as peptone, as substrate. Because it exhibits its maximum activity in the further hydrolysis of peptide nitrogen, this enzyme is referred to as peptonase, a term indicative of its action on peptone.

The intracellular peptonase of pneumococcus hydrolyzes 30 to 40 per cent of the peptide nitrogen in peptone substrates to amino nitrogen. The peptonase activity of the bacterial substance is striking in its intensity. Weight for weight the substance hydrolyzes peptone several times as rapidly as the most active commercial samples of pancreatic preparations. The zone of its optimum activity is pH 7 to 7.8, similar to that of trypsin and erepsin, and corresponds to the optimum reaction for growth of pneumococcus. The absence of activity at a pH below 4.5 indicates the absence of pepsin. Bile salts, as well as bile itself, effect solution of pneumococci, and enzymes prepared by dissolving the cell bodies in solutions of sodium choleate manifest an equal degree of activity. The thermostability of the intracellular peptonase is greater than the heat resistance of pneumococcus. The enzyme is, however, sensitive to heat; an exposure of 10 minutes at 100°C. destroys its activity. Dissolved in undiluted ox bile the enzyme retained about 40 per cent of its activity over a period of 6 weeks. A direct proportionality has been shown to exist between the rate of hydrolysis and the concentration of the enzyme in the digestion mixture.

In bacteria-free filtrates of pneumococcus enzymes are demonstrable only when growth of the bacteria has progressed to the phase in which cell disintegration begins and liberation of the intracellular sub-

stances into the culture medium occurs. During the early stages of growth of pneumococcus, when the organisms are multiplying at their maximum rate and little or no cell death is occurring, enzymes cannot be detected in culture filtrates. This evidence indicates that the enzymes studied are intracellular in character and belong to the class known as endoenzymes.

As far as is known for bacteria, solubility in bile is peculiar to pneumococcus alone. The mechanism of this reaction is not fully understood. Pneumococci exposed to an acidity equivalent to or greater than pH 5 are not only rapidly killed but rendered completely bile-insoluble. The endoenzymes derived from pneumococcus, on the other hand, are little influenced in their subsequent activity by previous exposure for 2 hours to a reaction corresponding to the acid death-point of the bacterial cell. Similarly pneumococci rapidly succumb on short exposure to a temperature of 52°C. and the heat-killed organisms are no longer soluble in bile. Exposure of the proteolytic enzyme, however, to a temperature corresponding to the thermal death-point of pneumococcus, causes only slight retardation of its hydrolyzing power.

These facts, apart from their significance in a study of the nature of the endoenzymes, are of interest in interpreting the possible relation of these active intracellular substances to the mechanism of bile solubility of pneumococcus. From these limited observations, it does not appear likely that bile solubility is the result of the action of enzymes, of which bile serves as an activator, for agents, both chemical and physical, which render the cell insoluble in bile, exert in a similar concentration only slight inhibition on the intracellular enzymes.

Rosenow found that extracts of virulent pneumococci possessed the power to split foreign proteins such as those present in ascites meat broth, while extracts of non-virulent organisms showed no digestion. The observations recorded in this paper on the relation of virulence to enzyme activity of pneumococcus are too limited to warrant any final judgment. However, under the experimental conditions, loss of virulence of the organism was not associated with a corresponding loss of enzyme activity. Pneumococci with virulence differing by a ratio of 1,000,000 to 1 showed quantitatively identical proteolytic power. In further elucidation of this problem it would be of interest

not merely to compare differences in enzymic activity with variations in virulence of the same strain, but also to contrast the relative potency of enzyme preparations from pathogenic pneumococci of the disease-producing types with the activity of similar preparations from the more saprophytic varieties of little or no virulence.

SUMMARY.

1. Pneumococci contain an intracellular enzyme or enzymes which (*a*) hydrolyze to some extent intact protein and (*b*) hydrolyze with striking avidity peptones. The optimum reaction for hydrolysis is pH 7 to 7.8, which also represents the optimum for the growth of pneumococcus. For convenience the terms "protease" and "peptonase" have been used, but no assumption is made as to whether the two actions, proteolysis and peptolysis, are due to two separate enzymes or are two activities of the same enzyme.

2. Solutions of intracellular substance of comparable enzymic activity may be prepared by dissolving the bacteria in bile, in sodium choleate, or by mechanical and autolytic disintegration of the cell.

3. The rapidity with which peptone is hydrolyzed is proportional to the concentration of the enzyme.

4. Heating the enzyme for 10 minutes at 100°C. destroys its activity.

5. Increasing the acidity to pH 5, the acid death-point of pneumococcus, suspends activity but does not destroy the enzyme, for activity is restored by readjustment to pH 7.8.

6. Attenuation of virulence to 1/1,000,000 of the original virulence had no measurable quantitative effect on the enzyme activity.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

II. Lipolytic Enzymes: Esterase.

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In the preceding paper (1) are recorded the facts so far obtained in a study of the proteolytic enzymes of pneumococcus. It has been shown that bile solutions of pneumococci, extracts obtained by disintegration of the organisms in phosphate mixtures, and sterile filtrates of autolyzing broth cultures possess the power to hydrolyze peptones and to a less extent certain intact proteins. In the present paper evidence will be presented that pneumococci possess also an endolipase of marked activity. The intracellular nature of this enzyme, the influence of age and hydrogen ion concentration on its activity, its thermostability, and relation to virulence and to the mechanism of bile solubility will be discussed.

In a monograph on bacterial enzymes, Fuhrmann (2) (1907) summarizes the work of earlier investigators on the occurrence of lipases in different species of microorganisms. In a review of the literature no reference has been found to work on the lipase of pneumococcus. Various methods for the demonstration of lipolytic activity of bacteria have been used. By a simple plate method, in which the zone of reaction about colonies of bacteria growing in the presence of certain test substances such as fat could be observed, Eijkman (3) detected lipolytic action in a number of organisms, including *Staphylococcus aureus*, *B. pyocyaneus*, *B. prodigiosus*, and *B. fluorescens*. Studying the lipase of *B. tuberculosis* and other bacteria, Wells and Corper (4), by testing the killed bacterial substance on esters and fats, demonstrated the presence of lipolytic enzymes in organisms which by the plate method apparently possessed no visible fat-splitting power. They further showed that sterile unheated emulsions of *B. tuberculosis*, while not actively lipolytic, possess enzymes capable of slowly hydrolyzing esters. Kendall, Walker, and Day (5) have demonstrated the occurrence of a soluble lipase in broth cultures of a variety of acid-fast organisms including tubercle bacilli of the human, bovine, and avian types. These authors found that the organisms, during the period of active growth, excrete a soluble lipase which occurs free in the medium.

Kendall and Simonds (6) have shown that sterile filtrates of plain and dextrose broth cultures of typhoid bacilli contain an esterase, capable of liberating acid from ethyl butyrate. The bacteria separated from the filtrates, however, showed but little esterase activity.

EXPERIMENTAL.

Methods.

Kastle and Loevenhart (7) have shown the advantage of using the lower esters, tributyrin and ethyl butyrate, in studying lipase activity. A preliminary experiment showed that when pneumococci are dissolved in bile the resultant solution contains an enzyme that splits both these esters. In the experiments to be recorded tributyrin has been used throughout as the substrate in the study of the intracellular lipase.

In determining lipase activity it has been customary to adjust the fat or ester substrate to approximate neutrality, with phenolphthalein as indicator, and then by titration to determine the amount of acid yielded by enzyme action. In the present study, however, it seemed more important to establish the optimum hydrogen ion concentration for action of the lipase, and then to maintain this reaction by the use of suitable buffer solutions. This buffered substrate maintains optimum conditions for enzyme action with a minimum of inhibition due to the acid products of hydrolysis. The amount of acid split off from the ester is determined by the amount of alkali required to readjust the digestion mixture to the initial reaction. It may also be calculated as the amount of acid required to change the buffered digestion mixture from the initial to the final hydrogen ion concentration. In the following experiments the ester was emulsified in 0.1 M phosphate solution of desired pH.

The method of preparing the enzyme solution, by dissolving the bacterial cells in sterile bile, was the same as that recorded in the experiments on the proteolytic enzymes of pneumococcus.

In no instances were antiseptics used as preservatives in the digestion mixtures. Sterility of all enzyme-substrate emulsions was proved by subculture.

Presence of an Intracellular Lipase and the Influence of Hydrogen Ion Concentration on Its Activity.

Experiment 1. (*a*) *Preparation of Enzyme.*—The bacterial residue from 4 liters of 18 hour plain broth culture of Pneumococcus Type II (No. F 208) was washed in isotonic salt solution, taken up in 20 cc. of sterile, undiluted ox bile, and held over night in the ice box. A portion of this enzyme solution was inactivated by heat.

(*b*) *Preparation of Substrate.*—2 per cent tributyrin (Kahlbaum) was emulsified in 0.1 M phosphate solution covering the range of pH values 4.9 to 7.8.

In carrying out the experiment 0.2 cc. of tributyrin was added to 10 cc. of sterile phosphate solution at the indicated reaction, and the mixtures were shaken until a fine emulsion was obtained. Three tubes were prepared at each reaction. 1 cc. of active enzyme solution was added to the first tube; 1 cc. of the same solution inactivated by heat to the second; and to the third 1 cc. of the undiluted bile used in preparing the bacterial solution. The tubes were then placed at 37°C. for 72 hours. The results of these experiments are given in Table I and are represented graphically in Text-fig. 1.

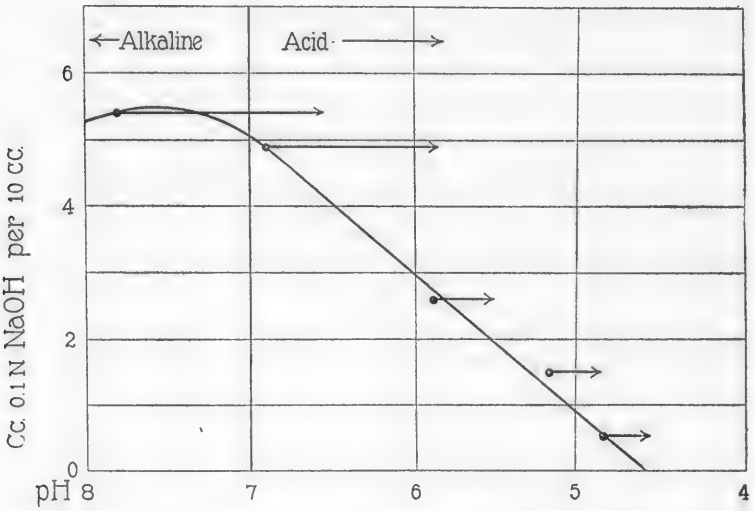
TABLE I.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Lipase of Pneumococcus.

Initial reaction of digestion mixture.	Hydrogen ion concentration after 72 hrs. at 37°C.			10 cc. of mixture containing active enzyme readjusted with 0.1 N sodium hydroxide.	
	10 cc. of 2 per cent tributyrin + 1 cc. of				
	Bile.	Inactive enzyme.	Active enzyme.	To initial pH.	Amount required.
pH	*pH*	*pH*	*pH*	*pH*	*cc.*
4.9	4.9	4.9	4.6	4.9	0.3
5.2	5.2	5.2	4.9	5.2	1.5
5.9	5.9	5.9	5.5	5.9	2.3
6.9	6.9	6.9	5.8	6.9	4.9
7.8	7.8	7.8	6.5	7.8	5.4

The facts brought out in Experiment 1 demonstrate that within the pneumococcus cell there exists a markedly active lipase, or esterase. The acid formed from 10 cc. of 2 per cent tributyrin was equivalent to 5.4 cc. of 0.1 N alkali, or a normality of about 0.05 N butyric acid. The maximum activity of this esterase occurs at a reaction of about pH 7.8 and progressively decreases with increase in acidity. This

optimum reaction corresponds closely with that of the intracellular peptonase, and coincides with the optimum hydrogen ion concentration for growth of pneumococcus.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular lipase of pneumococcus. The arrows indicate the extent of the reaction change.

Intracellular Nature of the Pneumococcus Lipase.

In the preceding paper it was shown that in filtrates of broth cultures of pneumococcus proteolytic enzymes were demonstrable only in the later phases of growth. Their appearance free in the culture medium coincided with the dissolution of the bacterial cells. In the early stages of growth, however, when cell multiplication is occurring at a maximum rate no enzymes are demonstrable in the culture filtrate. These facts indicate the intracellular nature of the proteolytic enzyme. Similarly it is shown in the following experiment that during the early phases of growth no lipase is present in cell-free filtrates. That the organisms were actively growing is evidenced from the change in reaction of the culture from pH 7.8 to 7.4.

Experiment 2.—20 cc. of the Berkefeld filtrate of a 5 hour culture of No. F 208 (the same preparation that was used in Experiment 8 in the preceding paper) were divided into two 10 cc. portions, one of which was autoclaved; 0.1 cc. of tributyrin was then added to each and the tubes were incubated for 48 hours. The results are given in Table II.

TABLE II.

Absence of Lipase in Culture Filtrates of Pneumococcus during the Period of Active Growth.

Filtrates from a 5 hour broth culture of Pneumococcus Type II.

Sterile filtrate.	Tributyrin.	Hydrogen ion concentration of filtrate.	
		Before incubation.	After incubation.
cc.	*cc.*	*pH*	*pH*
10, unheated.	0.1	7.4	7.4
10, heated.	0.1	7.3	7.3

From Table II it is evident that the lipase, like the peptonase, is an intracellular substance liberated on disintegration of the bacterial cell.

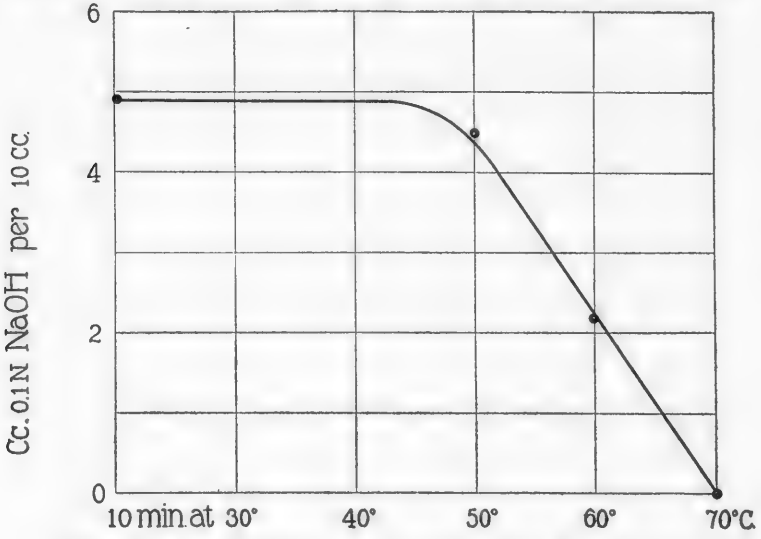
Effect of Heat on the Intracellular Lipase of Pneumococcus.

Experiment 3.—The thermostability of an enzyme in solution is influenced by the reaction of the medium in which it is dissolved and the length of exposure to the unfavorable temperature. In the present instance the enzyme was dissolved in bile, the solution adjusted to the optimum reaction for enzyme activity, pH 7.8, and subjected for 10 minutes in a water bath to the temperatures indicated.

The enzyme solution was the same as that used in Experiment 1 and was prepared from Pneumococcus Type II (No. F 208). 1 cc. portions of dissolved enzyme were carefully placed in sterile tubes and completely immersed in a water bath at the given temperature for 10 minutes. The tubes were immediately cooled to room temperature and to each were added 10 cc. of 2 per cent of tributyrin in 0.1 M phosphate solution, pH 7.8. After 24 hours at 37°C. the amount of acid split off from the ester by enzyme action was determined as in Experiment 1. The results are graphically presented in Text-fig. 2.

Variations in heat susceptibility of bacterial enzymes have been observed by many investigators. Söhngen (8), in studying the process of fat-splitting by bacteria, describes a lipase which resists a tempera-

ture of 100°C. for 5 minutes. The thermostability of the lipase of acid-fast bacteria has been noted by Wells and Corper, and by Kendall, Walker, and Day. These authors point out that heating to 100°C. for 15 minutes had little effect on the activity of the enzyme. Resistance of lipases in general to high temperatures is unusual; the various plant and tissue lipases in solution are as a rule inactivated by temperatures of 60–70°C. From Text-fig. 2 which illustrates the



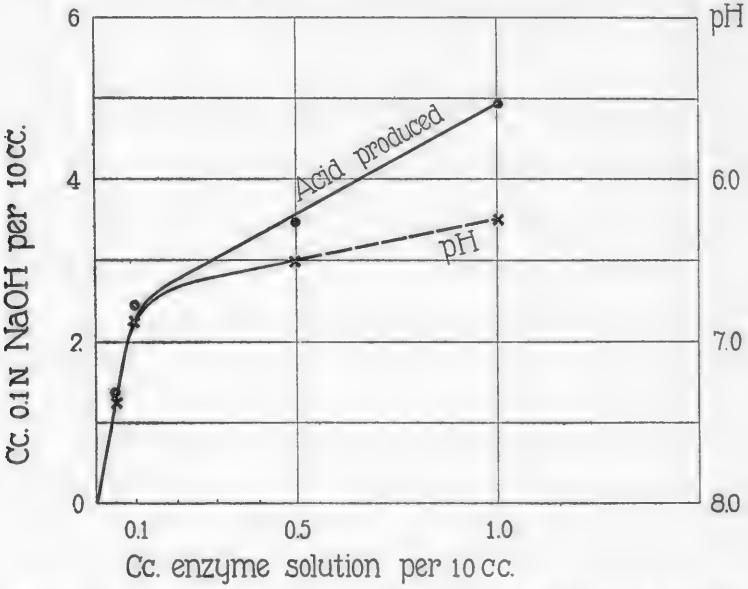
TEXT-FIG. 2. Heat stability of the intracellular lipase of pneumococcus.

effect on the pneumococcus lipase of exposure to various temperatures, it is evident that the dissolved enzyme suffers progressive loss of activity at temperatures above 50°C. and complete destruction at 70°C. for 10 minutes. According to Sternberg (9) the thermal death-point of pneumococcus is 52°C. for 10 minutes. The thermostability of the intracellular lipase under the conditions of this experiment, therefore, is somewhat greater than the heat resistance of the living organism.

Effect of Concentration of Enzyme on the Activity of the Intracellular Lipase of Pneumococcus.

If the ester-splitting property of bile solutions of pneumococci is enzymotic in nature, the rate of acid production should be proportional to the concentration of enzyme present. That this is the case is shown in the following experiment.

Experiment 4.—The enzyme solution, the same preparation used in the preceding experiment, was diluted with bile so that 1 cc. contained from 0.05 to 1 cc. of the original enzyme solution. These graduated quantities were added to 10 cc. of substrate consisting of 2 per cent tributyrin emulsified in 0.1 M phosphate solution of pH 7.8. The enzyme-substrate mixtures were incubated at 37°C. for 24 hours and the amount of acid hydrolysis was determined by the method outlined. The results are given in Text-fig. 3.



TEXT-FIG. 3. Influence of concentration of enzyme on the activity of the intracellular lipase of pneumococcus.

It is evident from the curves presented, that the rate of acid production by the action of the intracellular lipase on tributyrin is

directly proportional to the concentration of enzyme. Furthermore, as the enzyme concentration approaches the maximum, the amount of acid liberated by ester cleavage becomes so great that the resulting acidity is of itself sufficient to retard further enzyme action.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Experiment 5. Preparation of Enzyme.—Two subcultures of the same strain of Pneumococcus Type II (No. F 208) were used. One of these, designated No. F 208 "B," the virulence of which had been greatly attenuated by cultural methods, failed to kill white mice in doses of 1 cc. of broth culture. The other, No. F 208 A, representing the original strain, the virulence of which had been preserved, was invariably fatal to these animals in doses of 0.000001 cc. injected intraperitoneally. The washed bacterial residues from 1,500 cc. of 15 hour plain broth cultures of these two organisms were collected and each was dissolved in 15 cc. of undiluted, sterile ox bile. The respective solutions were held over night in the ice box to ensure complete plasmolysis and were then tested for sterility on blood agar and in broth. The control enzyme solutions were inactivated by heat.

1 cc. portions of the active and inactivated enzyme preparations were added to tubes containing 10 cc. of 2 per cent tributyrin emulsified in sterile 0.1 M phosphate solution at pH 7.8. The digestion mixtures were then incubated for 24 hours at 37°C. Determinations of the hydrogen ion concentrations before and after incubation and of the amounts of acid produced in each instance are given in Table III.

TABLE III.

Relation of Virulence of Pneumococcus to Activity of the Intracellular Lipase.

Pneumococcus Type II.		Enzyme.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.	Increase due to enzyme action.
Strain.	Minimum lethal dose for mice.		Initial.	Final.		
	cc.		*pH*	*pH*	*cc.*	*cc.*
F 208 "B"	Greater than 1	Active.	7.8	6.7	3.95	3.60
		Inactive.	7.8	7.7	0.35	
F 208 A	0.000001	Active.	7.8	6.7	3.75	3.45
		Inactive.	7.8	7.7	0.30	

Under the conditions of Experiment 5, in which were compared the relative potencies of the endoenzymes of an avirulent and of a virulent culture of the same strain of pneumococcus, loss of virulence was not associated with loss of enzyme activity.

Effect of Exposure to Acid Reaction on the Subsequent Activity of Pneumococcus Lipase.

Lord and Nye (10) have shown that pneumococci are rapidly killed at a hydrogen ion concentration of about pH 5.1. This acid deathpoint has been found both by these observers and by the present authors to correspond to the final reaction of broth cultures of pneumococcus when grown in the presence of sufficient carbohydrate. To determine whether this reaction is fatal to both organism and enzyme alike and whether this correlation has any significance in the mechanism of bile solubility the following experiment was performed.

Experiment 6.—1 cc. of active enzyme solution prepared as in Experiment 1 was added to 9 cc. of 0.1 M acid potassium phosphate solution of pH 4.6 (resulting reaction pH 5) and incubated at 37°C. for 2 hours. Then 0.83 cc. of N sodium hydroxide (calculated and verified on separate 9 cc. samples) was added to bring the acid enzyme solution to pH 7.8. After this readjustment of reaction 0.2 cc. of tributyrin was added as substrate, and the enzyme-substrate mixture incubated for 42 hours at 37°C. To serve as a control on activity, 1 cc. of the same enzyme solution, untreated, was added directly to 10 cc. of 0.1 M phosphate solution at pH 7.8 containing 2 per cent tributyrin (Table IV).

TABLE IV.

Effect on Lipase of Exposure to Acid Reaction.

Pneumococcus Type II.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
	Initial.	Final.	
	pH	*pH*	*cc.*
Untreated enzyme.	7.8	6.3	4.93
Acid-treated and readjusted.	7.8	6.4	3.80

From Table IV it is evident that after neutralization the activity of the pneumococcus lipase is little influenced by previous exposure

to a reaction of pH 5. The bile insolubility of pneumococci after similar acid treatment is apparently not attributable to the death of the lipase, but is possibly referable to changes in the cell protoplasm.

Effect of Age on the Activity of the Intracellular Lipase of Pneumococcus.

Experiment 7.—Enzyme solutions prepared from pneumococci by extraction in phosphate solution as described in the preceding paper possessed lipase activity entirely comparable to that of enzyme solutions prepared by the bile method. These preparations were still active after preservation for 7 weeks, as is shown in Table V.

TABLE V.

Age Stability of Lipase.

Pneumococcus Type II.	Age.	Hydrogen ion concentration.		Amount of 0.1 N sodium hydroxide per 10 cc. required to readjust to initial pH.
		Initial (0.1 M phosphate).	Final.	
	wks.	*pH*	*pH*	*cc.*
No. F 208	7	7.8	7.1	4.9
" II	3	7.8	6.9	6.5

DISCUSSION.

When pneumococci are dissolved in bile or extracted by the methods described, the resultant solution possesses, in addition to the proteolytic activity recorded in the preceding paper, a lipase (esterase) as measured by its power to split off acid from tributyrin.

Since a number of investigators (Hewlett (11), Magnus (12), and Loevenhart and Souder (13)) have shown that bile and bile salts not only do not interfere with lipase activity, but on the contrary accelerate the reaction, it was to be expected that the use of bile in effecting solutions of pneumococci would serve as an ideal method for demonstrating lipase activity. Bile, however, is not essential to the reaction, since extracts prepared by other methods exhibit comparable activity.

This esterase manifests its maximum activity at a hydrogen ion concentration of about pH 7.8, which is the optimum reaction for initiating growth of pneumococcus. The lipolytic activity of this enzyme progressively diminishes with increasing acidity until at about

pH 5 further hydrolysis ceases. That the point of acid extinction of esterase activity corresponds closely with the acid death-point of the living pneumococcus reveals another interesting correlation between cellular function and enzyme action. The rate of acid liberation from tributyrin during the initial stage of the action of the intracellular lipase is directly proportional to the concentration of enzyme. In the later phases of the reaction, the acid liberated by ester cleavage is sufficient in itself to retard further hydrolysis.

That the pneumococcus lipase is intracellular in nature is evidenced by the fact that it is present in maximum amount in bile solutions of washed bacterial cells, but cannot be demonstrated in culture filtrates during the period of active growth of the organism. The thermostability of the endolipase in solution is greater than the heat resistance of the living pneumococcus. After 10 minutes exposure in a water bath at temperatures greater than 50°C., the dissolved enzyme suffers progressive loss in activity until at 70°C. complete destruction results. Enzyme solutions preserved at refrigerator temperature retain their activity for weeks.

As to the possible relation of the activity of endoenzymes of pneumococcus to virulence of the living cell, the observations recorded in these studies are too limited to warrant discussion. Comparison of the relative potency of the endoenzymes of avirulent and virulent cultures of the same strain of pneumococcus has shown, however, that under the experimental conditions defined, loss of virulence was not associated with loss of enzymic activity.

Pneumococci exposed to a reaction corresponding to the acid death-point of the cell, that is, an acidity equivalent to or greater than pH 5, are thereby rendered insoluble in bile. This bile insolubility of pneumococci after acid treatment persists upon neutralization of the acid and even after the cells have been removed, washed, and resuspended in a neutral medium. In view of the possibility that the mechanism of bile solubility might in some way be related to the endolipase, in which instance the bile salts might function as coenzyme, or activator, it seemed pertinent to determine the effect of exposure to acid reaction on the subsequent activity of the enzyme itself. Since it has been shown, however, that upon neutralization the activity of the lipase is little influenced by previous exposure to a reaction of

pH 5, an acidity which kills the living cell, the phenomenon of bile insolubility of pneumococci after similar acid treatment is apparently not attributable to destruction of the endoenzyme.

SUMMARY.

1. Pneumococci contain an intracellular enzyme of marked lipolytic activity as measured by the acid liberated by its action on tributyrin.

2. Enzyme-containing solutions may be prepared by dissolving pneumococci in bile, or by extraction by other means.

3. The optimum reaction for maximum activity of the endolipase is about pH 7.8, which coincides with the optimum hydrogen ion concentration for growth of pneumococci.

4. Heating the enzyme for 10 minutes at 70°C. destroys its activity.

5. Attenuation of virulence of pneumococcus had no measureable effect on enzyme activity.

6. The possible relation of the endolipase to the mechanism of bile solubility is discussed.

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STUDIES ON THE ENZYMES OF PNEUMOCOCCUS.

III. Carbohydrate-Splitting Enzymes: Invertase, Amylase, and Inulase.

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The two preceding papers (1, 2) on the enzymes of pneumococcus have dealt with the proteolytic and lipolytic activities of extracts of the bacterial cells, and of sterile filtrates of cultures. It has been pointed out that enzymes capable of actively hydrolyzing various substrates exist preformed within the cell and that by suitable methods they can be obtained free in solution and their action studied independently of the living organism.

The avidity with which pneumococcus attacks certain carbohydrates is manifest in the accelerated growth and increased acid production of organisms cultivated in the presence of these substances. Acids are produced in culture media from starches and glucosides, as well as from mono- and disaccharides. It has been customary to assume that these fermentation reactions are the summation of the action of several enzymes, first, the hydrolysis of the disaccharide or starch to monosaccharide, and secondly, the production of acid from the monosaccharide.

It was of interest, therefore, to determine whether the bile solutions of pneumococci which were known to contain protein- and fat-splitting enzymes also contained either or both groups of carbohydrate enzymes. Tests upon glucose, saccharose, starch, and inulin substrates were carried out with enzyme solutions containing peptonase and lipase prepared by dissolving pneumococci in bile.

The simple expedient of dissolving the bacterial cells in bile and testing the resultant solution for the presence of enzymes, a method admirably adapted to the determination of proteolytic and lipolytic activity, was found unsatisfactory for studying the enzymes attacking

carbohydrates, since bile in the concentration necessary to effect bacterial solution completely inhibits the activity of the enzymes converting sugar and starch. Consequently the method described below was adopted, by which disintegration of the pneumococcal cells is effected by suspending them in balanced phosphate solution at pH 6.2 and hastening physical disruption by repeated freezing and thawing of the bacterial suspension. The enzymes liberated from the bacterial bodies in this manner, as will be shown in the following experiments, are capable of hydrolyzing sucrose, starch, and inulin, the three test substances chosen for the typical reactions of invertase, amylase, and inulase.

EXPERIMENTAL.

Bacteriological Methods.

Preparation of Enzyme Solution by the Acetone Method.—Because of the fact that precipitation with acetone has been a satisfactory method for preparing and purifying many types of enzymes, an attempt was made to obtain an active carbohydrate-splitting enzyme from pneumococcus by the following procedure.

The residue of 2 liters of an 18 hour broth culture of pneumococcus was taken up in two 10 cc. portions of sterile distilled water, and each portion was poured into 150 cc. of acetone. After standing over night the supernatant solutions were removed from the precipitates which were then allowed to dry. Cultural examination showed that the organisms had been killed by acetone precipitation, although they remained intact and were Gram-positive.

One portion of the dry residue was shaken up with 10 cc. of 0.1 M phosphate at pH 7.4; the other was treated with 2 cc. of 0.1 N sodium hydroxide, allowed to stand for several hours, then neutralized with 0.1 N hydrochloric acid to pH 7.4, and diluted to 10 cc. Each portion was tested for lipase, for peptonase, and for invertase. The tests were all negative.

Preparation of Enzyme Solution at pH 6.2.—Previous work has shown that growth of pneumococcus cannot be initiated at a reaction more acid than pH 6.8 (3), and that disintegration of the bacterial

cell occurs most rapidly at about pH 6.2. Moreover, although the subsequent activity of the intracellular lipase and peptonase at optimum reactions is not materially influenced by temporary exposure to an acidity as great as pH 5, they show much less activity at pH 6.2 than at a neutral or slightly alkaline reaction. It seemed probable therefore, that disintegration of the pneumococcus cell, under conditions at which the autolytic processes are at a minimum and at which neither initial growth of the organism nor destruction of known enzymes occurs, might liberate the intracellular carbohydrate-splitting enzymes. Such conditions were obtained in the following manner.

The washed bacterial residue from 1.5 liters of an 18 hour plain broth culture of pneumococcus was taken up in 15 cc. of 0.1 M phosphate solution of pH 6.2, and placed in the ice box until intact bacterial cells could no longer be found under the microscope. After the solution had been proved sterile, it was tested for the presence of known enzymes. The lipase activity of this solution was comparable to that obtained with the bile solution previously used.

Preparation of Enzyme Solution by Cytolysis with Alternate Freezing and Thawing.—This method differed from the preceding only in that the disintegration of the cell was hastened by repeated freezing and thawing. An ice-salt mixture of about −22°C. was used.

Sterility Controls.—In addition to the sterility tests on the enzyme solution, the final enzyme-substrate mixtures were tested by transferring 0.1 cc. to 5 cc. of broth and incubating for 48 hours.

Chemical Methods.

Preparation of Substrates. Sucrose.—A 4 per cent sucrose solution was sterilized in boiling water for 20 minutes. 25 cc. of this sterile 4 per cent sugar solution were then added to 25 cc. of sterile phosphate solution of the desired pH.

Glucose.—Prepared in the same manner as sucrose.

Starch.—A 2 per cent and a 0.2 per cent suspension of Kahlbaum's rice starch in 0.1 M phosphate solution of pH 7.4 were autoclaved for 20 minutes at 15 pounds pressure.

Inulin.—A 2 per cent inulin solution in 0.1 M phosphate at pH 7.4 was sterilized as above.

Phosphate Solutions.—The 0.1 M phosphate solutions for the range pH 5 to 8.3 were prepared from potassium acid phosphate and sodium phosphate (Na_2HPO_4) according to Sörensen's tables. For the more acid range, mixtures of 0.1 M potassium acid phosphate and 0.1 N hydrochloric acid were used. In all the experiments the appropriate corrections were made for the effect on the reaction of the enzyme solution. All phosphate solutions were sterilized by autoclaving for 20 minutes at 15 pounds pressure.

Hydrogen Ion Concentration.—The hydrogen ion concentration was ordinarily determined by the colorimetric method. For the range of acidity greater than pH 4.5 and for frequent controls of the colorimetric standards, the electrometric method, using Clark's rocking electrodes (4), was employed.

Determination of Reducing Sugar.—Qualitative tests for reducing sugar were carried out by using 5 cc. of Benedict's qualitative solution and 0.5 cc. of test solution and boiling for 10 minutes.

Quantitative sugar determinations were made with either Benedict's quantitative titrating method, by determining the rotation of the solution, or by the gravimetric copper method.

Carbon Dioxide.—Determinations were made with Van Slyke's apparatus (5).

Amylase Action.—Hydrolysis of starch to dextrins was determined by means of the iodine color test. 1 cc. of the solution was diluted with 2 cc. of water and 0.2 cc. of a dilute iodine solution (about $\frac{N}{150}$) added. Hydrolysis of the starch or dextrin to reducing sugar was determined as indicated above.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Experiment 1.—A solution of Pneumococcus Type II prepared as outlined above was added to a series of tubes containing saccharose, inulin, starch, glucose, and glucose-peptone mixture. The glucose-peptone solution was used with the idea that the nitrogen of the peptone might accelerate glucose hydrolysis. The tubes were incubated for 48 hours at 37°C. The solutions were then analyzed as described. The results are given in Table I.

Experiment 2.—This experiment differed from the preceding one in that the suspension of pneumococci was frozen and thawed five times. The enzyme solution was held at ice box temperature (4°C.) for 16 days until culture controls in blood broth no longer showed the presence of viable organisms.

In addition to the substrates used in the preceding experiment two additional control tubes were included, one containing 10 cc. of a 2 per cent saccharose solution in 0.1 M phosphate solution plus 1 cc. of bile, and the second containing 0.2 per cent glucose. Since slight glucose fermentation might have been masked by an excess of the sugar, the dilute solution was included in this series. The results are presented in Table II.

TABLE I.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by cytolysis at pH 6.2.

Substrate in 0.1 M phosphate solution.	Final hydrogen ion concentration.		Qualitative determinations with Benedict's solution.		Color with iodine.		Rotation.		Carbon dioxide content per 2 cc.	
	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.	Inactive.	Active.
	pH	*pH*							*cc.*	*cc.*
Saccharose, 2 per cent.	7.4	7.4	−	++++			2.7°	1.36°	0.075	0.080
Glucose, 2 per cent.	7.4	7.4	++++	++++					0.075	0.075
Glucose, 1 per cent, in 1 per cent peptone.	7.4	7.4	++++	++++					0.075	0.090
Inulin, 2 per cent.	7.4	7.4	−	++						
Starch, 2 per cent.	7.4	7.4	−	+	Blue.	Lavender.	0°	0.2°		
Starch, 0.2 per cent.	7.4	7.4	−	+	"	Vanishing light lavender.				
Tributyrin,* 1 per cent.	7.8	6.3								

* Tributyrin was used as a control of enzyme activity.

It is evident from Tables I and II that pneumococcus contains enzymes that hydrolyze starch to dextrins (amylase), hydrolyze inulin (inulase), and invert saccharose to reducing sugars (invertase). The microorganism appears, therefore, to contain enzymes capable of hydrolyzing complex carbohydrates into simple sugars. Bile

completely inhibits the hydrolysis; whether this is due to destruction of the enzyme or to inhibition of its action has not been determined. This fact explains the failure to detect the carbohydrate enzymes in the bile solution used for the study of the peptonase and lipase of pneumococcus. On the other hand, the peptonase and lipase are as active in the solution obtained by the method described as in the bile solutions.

All attempts to demonstrate an enzyme capable of fermenting glucose or producing acid from glucose were unsuccessful.

TABLE II.

Action of Intracellular Enzymes of Pneumococcus on Carbohydrates.

Enzyme solution prepared by alternate freezing and thawing.

Substrate in 0.1 M phosphate solution.	Hydrogen ion concentration.		Qualitative determinations with Benedict's solution.	Color with iodine.	Carbon dioxide content per 2 cc.
	Initial.	Final.			
	pH	*pH*			*cc.*
Glucose, 2 per cent	7.3	7.3	++++		0.09
" 0.2 per cent	7.3	7.3	+++		
Starch, 2 per cent	7.3	7.3	++	Lavender.	
" 0.2 per cent	7.3	7.3	+	Vanishing lavender.	0.07
" 0.2 " " (control)	7.3	7.3	−	Blue.	
Inulin, 2 per cent	7.3	7.3	+		
Saccharose, 2 per cent	7.3	7.3	++++		0.07
" + 1 cc. of bile	7.3	7.3	−		
Control	7.3	7.3	−		
Tributyrin,* 1 per cent	7.8	6.3			

* To control enzyme activity.

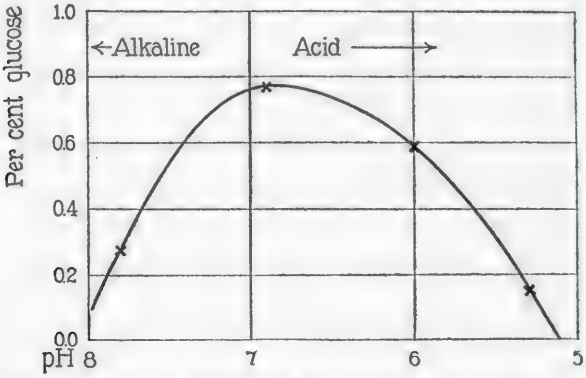
Intracellular Nature of the Carbohydrate-Splitting Enzymes.

Experiment 3.—Tests for invertase, amylase, and for a glucose-fermenting enzyme in the filtrate from a young actively growing culture of pneumococcus were carried out exactly as in the case of lipase and peptonase. Saccharose, starch, and glucose to make 1 per cent solutions were added to the sterile filtrate from a 5 hour culture of Pneumococcus Type II, and the mixture was incubated for 48 hours at 37°C. The mixtures were tested as in the preceding experiment and no evidence of enzyme action was demonstrable. Since in the solutions of pneumococci

obtained by the method described there are active carbohydrate-splitting enzymes, their absence in the filtrates of cultures during the early phase of growth, before cell disintegration has occurred, indicates that, like lipase and peptonase, these enzymes are intracellular in nature.

Influence of Hydrogen Ion Concentration on the Activity of the Intracellular Invertase of Pneumococcus.

The preceding experiments have demonstrated the presence of several carbohydrate-splitting enzymes. It now seemed desirable to determine the influence of the hydrogen ion concentration on their activity.



TEXT-FIG. 1. Influence of hydrogen ion concentration on the activity of the intracellular invertase of pneumococcus.

Experiment 4. (*a*) *Preparation of Enzyme Solution.*—The solution was prepared as described in preceding experiments.

(*b*) *Preparation of Substrate.*—The substrates were prepared as outlined under Methods. 1 cc. of enzyme was added to 10 cc. of substrate solution; a duplicate tube without enzyme served as control for acid hydrolysis.

Both qualitative and quantitative reducing sugar determinations were made. The results are given in Table III and Text-fig. 1.

Optimum Hydrogen Ion Concentration for Amylase Action.

These experiments were carried out as in the case of invertase. Although quantitative results were not carried out, the iodine color indicates that the optimum reaction is about pH 7.

TABLE III.

Influence of Reaction on Invertase Action.

Enzyme preparation.	Hydrogen ion concentration.	Glucose determinations.		
		Qualitative determinations with Benedict's solution.	Quantitative determinations by Benedict's method.	Quantitative determinations by the gravimetric copper method.
	pH		*per cent*	*per cent*
No. D 39	7.7	++	0.1	
" D 39	6.9	+++	0.51	
" D 39	6.1	+++	0.34	
" D 39	5.2	+	0.1	
" D 39	4.8	−	0.0	
" F 208	7.8	+±		0.29
" F 208	6.9	+++		0.77
" F 208	6.0	++		0.59
" F 208	5.3	+		0.15
" F 208	4.85	−		

All controls negative.

TABLE IV.

Influence of Reaction on Amylase Action.

Enzyme. preparation.	Hydrogen ion concentration.	Qualitative determinations with Benedict's solution.	Color with iodine.
	pH		
No. D 39	8.2	−	Deep, fading blue.
" D 39	6.9	++	Red-lavender.
" D 39	6.1	+	Blue-lavender.
" D 39	5.1	−	Blue.
" D 39	4.8	−	
" F 208	7.8	±	Very pale lavender, fading instantly.
" F 208	6.9	++	Pale lavender, fading rapidly.
" F 208	6.0	++	Deep " " slowly.
" F 208	5.3	−	Faint change only.
" F 208	4.8	−	Blue as controls.

It is evident from Tables III and IV that the optimum hydrogen ion concentration for pneumococcus invertase and amylase is about pH 7.

DISCUSSION.

It is generally accepted that in the utilization of carbohydrates by living bacteria, hydrolysis of these complex substances is brought about through the action of enzymes. The fact has been recognized that enzymes capable of converting sucrose and starch may be found in fungi, especially in yeasts and moulds. Studies on the carbohydrate-splitting processes of invertase-producing bacteria have also been reported by numerous investigators. The earlier work of Fermi and Montesano (3), particularly, lists a number of microorganisms, in sterile cultures of which invertase activity was demonstrable. The isolation and study of carbohydrate-splitting enzymes apart from the living cell have not, so far as we have been able to find in the literature, been attempted in the case of pneumococcus.

The demonstration of the intracellular agents concerned in carbohydrate cleavage by pneumococcus was accomplished by a method through which the release of endoenzymes from the intact organism was effected by breaking down of the cell structure under conditions not injurious to the reactive substances themselves. That physical disruption of the cell membrane through alternate freezing and thawing is subsequently followed by autolytic processes is indeed likely; that the chemical changes brought about by autolysis under these conditions, however, exert but slight influence on the activity of the enzymes studied is evidenced by the avidity with which hydrolysis occurs, and the length of time during which potency is preserved.

From the data presented in this and the two preceding papers, it may be concluded that within the cell bodies of pneumococci there exist in addition to the endohemotoxin described by Cole (6), a series of intracellular enzymes. The proteolytic and lipolytic functions of this endoenzyme-complex have already been described. In addition, there may now be added the activity of the endoenzymes causing hydrolysis of carbohydrate substances, such as sucrose, starch, and inulin.

The optimum hydrogen ion concentration for the invertase and amylase of pneumococcus is about pH 6.8 to 7. This represents a reaction slightly less alkaline than that shown to be optimum for the activity of the peptonase and esterase. The reaction most favorable

for the activity of enzymes that attack carbohydrates is not the same even for those having similar activities. Sörensen (7) has shown that the optimum hydrogen ion concentration for the invertase of beer yeast is about pH 4.5. In studying the influence of hydrogen ion concentration on the enzymic activity of three typical amylases of different origin, Sherman, Thomas, and Baldwin (8) found that the starch-splitting enzymes of malt and *Aspergillus oryzæ* are both most active at an acid reaction (pH 4.4 to 4.8), while the pancreatic amylase reaches its maximum at pH 7. The carbohydrate-splitting enzymes of pneumococcus function best at a neutral reaction and are operative over a zone which corresponds closely to the reaction range of the living organism when grown in the presence of fermentable substances (9). Attempts to determine the presence of an enzyme or enzymes capable of fermenting dextrose and producing acid, an action characteristic of the growing cell, have been unsuccessful.

The invertase, amylase, and inulase of pneumococcus, like the proteolytic and lipolytic enzymes, are intracellular in nature and are found free in culture fluids only after cell disintegration has begun.

SUMMARY.

1. A method is described for the preparation of an active enzyme-containing solution of pneumococci, in which no living cells are present. These enzymes are capable of hydrolyzing sucrose, starch, and inulin.

2. The invertase and amylase of pneumococcus are active within the limits pH 5 to 8, with an optimum reaction of about pH 7. This reaction range corresponds closely with limiting hydrogen ion concentrations which define growth of the organism in the presence of carbohydrate.

3. These studies indicate that the enzymes described are not true secretory products of the living cell, but are of the nature of endoenzymes, since their activity can be demonstrated only when cell disintegration has occurred.

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EPINEPHRINE CONTENT OF THE SUPRARENAL GLANDS IN ANAPHYLAXIS.

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PLATE 89.

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Since the discovery of the pressor action of the internal secretion of the suprarenal glands by Oliver and Schäfer (1) many attempts have been made to assign to these glands a function of considerable importance, that of maintaining the normal tone of the vascular system in combating agencies which tend to lower the blood pressure and produce shock-like phenomena. The suprarenal glands were examined histologically in different conditions of shock with somewhat variable results. Bainbridge and Parkinson (2) found by staining methods that epinephrine entirely disappeared from the suprarenal glands in cases of death from acute infections, and in postoperative shock. Hornowski (3) likewise found, by staining methods, that the epinephrine disappears from the suprarenals in postoperative shock, and he believes the latter to be due to epinephrine depletion. Corbett (4) states that the epinephrine store of the suprarenals is depleted to the extent of from 75 to 3 per cent of the normal by various experimental procedures leading to shock. Cramer (5), on the other hand, using histochemical methods, found the suprarenals actually loaded with epinephrine granules, and actively secreting them into the central vein in cases of postoperative shock, at a time when the blood pressure was low and the temperature subnormal.

The epinephrine output of the suprarenals in various conditions of experimental shock has been investigated by Bedford (6), who found that the epinephrine content of adrenal blood was greatly augmented during shock, and this he believes to be associated with an increased activity of the glands, a mechanism to save the animal from shock and low blood pressure. Stewart and Rogoff (7), making quantitative estimations of the rate of output of epinephrine from the suprarenal glands in a given unit of time, found that this was not influenced by low blood pressure such as occurs after various procedures to induce shock.

The condition of the suprarenal glands in anaphylactic shock and during the state of sensitization does not seem to have been examined, either as regards pathological changes therein, or from the standpoint of their functional activity, although pathological changes in the liver, kidney, muscle, myocardium, spleen,

and blood vessels have been described in experimental acute and chronic anaphylaxis (Gay and Southard (8), Longcope (9), and Boughton (10)). In view of the alleged alterations of the epinephrine content and output of the suprarenal glands in various forms of surgical shock, it seemed probable that the suprarenal glands of animals in anaphylactic shock might show similar changes, especially so since the experiments of Hirschfeld and Hirschfeld (11) suggest the presence of a vasoconstricting substance in the blood of animals in anaphylactic shock. Furthermore, a more or less beneficial effect has been shown to be produced by the administration of epinephrine to animals in anaphylactic shock (Galambos (12)).

We therefore undertook to determine the epinephrine content of the suprarenal glands in animals sensitized to a foreign protein, as well as in those subjected to varying degrees of anaphylactic shock. Guinea pigs and rabbits were used in the experiments. The animals were sensitized by a subcutaneous or intraperitoneal injection of ox serum, and after an incubation period of at least 2 weeks they were reinjected with the antigen intravenously. Immediately after death or at the height of shock the suprarenal glands were removed,[1] weighed, and their epinephrine content was determined. We have also determined the epinephrine content of the suprarenals of sensitized, but not shocked animals. In the latter group the animals were killed at the height of sensitization by a blow on the neck. All the control animals were treated in the same manner.

The epinephrine estimations were made, for the most part, by the colorimetric method of Folin, Cannon, and Denis (13). We have not, however, used their uric acid standard, but have employed instead a freshly prepared solution of epinephrine hydrochloride made from the adrenalin of Parke, Davis and Company.

The results of the experiments are summarized in Table I, from which it will appear that the epinephrine content of the suprarenal glands in sensitized and in shocked animals does not materially differ from that of normal animals. The average epinephrine content of the suprarenals of normal guinea pigs was 0.073 mg. per 100 mg. of gland, that of sensitized guinea pigs 0.066 mg. per 100 mg. of gland, while that of guinea pigs dying in anaphylactic shock was 0.072 mg. per 100 mg. of gland. The epinephrine content of the suprarenals of normal rabbits averaged 0.30 mg. per 100 mg. of gland, that of a

[1] All operations were performed under ether anesthesia.

fully sensitized rabbit was found to be 0.27 mg. per 100 mg. of gland, while the average epinephrine content of rabbits in anaphylactic shock for periods varying from 2 minutes to 3 hours appeared to be 0.28 mg. per 100 mg. of gland.

TABLE I.

Epinephrine Content of the Suprarenals in Normal, Sensitized, and Shocked Animals Determined by the Colorimetric Method.

Experiment No.	Weight.	Condition.	Weight of suprarenals.	Epinephrine.	
				Total.	Amount per 100 mg. of gland.
		Guinea pigs.			
	gm.		*mg.*	*mg.*	*mg.*
1	410	Normal.	270	0.19	0.070
2	460	"	350	0.29	0.083
3	465	"	230	0.15	0.065
4	455	Sensitized.	290	0.18	0.062
5	468	"	270	0.19	0.070
6	385	Anaphylactic shock; died in 4 min.	160	0.13	0.081
7	450	" " " " 3 "	230	0.17	0.074
8	355	" " " " 4 "	180	0.09	0.050
9	455	" " " " 3 "	250	0.16	0.064
10	425	" " " " 4 "	280	0.20	0.072
11	378	" " " " 3 "	230	0.21	0.091
12	442	" " " " 4 "	220	0.16	0.073
13	363	" " " " 4 "	320	0.24	0.075
14	445	" " " " 4 "	330	0.22	0.067
		Rabbits.			
	kg.				
15	1.9	Normal.	320	1.27	0.39
16	1.9	"	150	0.33	0.22
17	2.0	Sensitized.	230	0.63	0.27
18	1.5	In anaphylactic shock 20 min.; killed.	100	0.37	0.37
19	1.5	Anaphylactic shock; died in 2 min.	100	0.25	0.25
20	1.7	" " " " 3 hrs.	280	0.63	0.23

The chemical method for estimation of epinephrine such as we employed in the above experiments may be open to objection, since substances other than epinephrine, notably uric acid, give similar color reactions. It was thought desirable, therefore, to confirm the

above findings by some other method, and a few experiments were made to determine the epinephrine content of the suprarenals of normal, sensitized, and shocked guinea pigs by the segment method of the surviving rabbit intestine after the method of Cannon and de la Paz (14) and Hoskins (15). The suprarenal glands were extracted with normal saline solution, and assayed for their epinephrine content against a freshly diluted solution of adrenalin hydrochloride 1:1,000 of Parke, Davis and Company. In some of the experiments (Nos. 26 to 29, Table II) the suprarenals were extracted, as for the

TABLE II.

Epinephrine Content of the Suprarenals in Normal, Sensitized, and Shocked Guinea Pigs Determined by the Biological Method.

Experiment No.	Weight.	Condition.	Weight of suprarenals.	Epinephrine.	
				Total.	Amount per 100 mg. of gland.
	gm.		*mg.*	*mg.*	*mg.*
21	400	Normal.	250	0.10	0.04
22	400	"	250	0.20	0.08
23	425	Sensitized.	270	0.10	0.04
24	425	"	300	0.12	0.04
25	404	In anaphylactic shock.	350	0.15	0.04
26	445	Anaphylactic shock; died in 4 min.	330	0.10	0.03
27	363	" " " " 4 "	320	0.10	0.03
28	442	" " " " 4 "	220	0.15	0.07
29	378	" " " " 4 "	230	0.08	0.03

colorimetric method, with 1 cc. of 0.1 N hydrochloric acid in 8.5 cc. of water, and heated to boiling. 0.5 cc. of 10 per cent sodium acetate was then added, and the whole filtered. The standard for these experiments was made in the same manner; *i.e.*, a solution was made to contain 1 mg. of epinephrine in 10 cc. containing 1 cc. of 0.1 N hydrochloric acid and 0.5 cc. of 10 per cent sodium acetate. This was further diluted with water, as required. The segment of rabbit intestine was always suspended in the same volume of Tyrode solution, oxygenated and kept at a constant temperature of 38°C. Fig. 1 illustrates the method.

The results of these experiments are presented in Table II. It will be seen that the epinephrine content of the suprarenals, as in the preceding experiments, runs practically the same in the sensitized and shocked animals as in the normal controls.

It is not desired to draw too far reaching conclusions from these experiments. They show clearly that the epinephrine store of the suprarenal glands is not materially altered by sensitization or by varying degrees of anaphylactic shock. Neither the epinephrine output nor the activity of the suprarenal medulla in sensitized and shocked animals, however, can be judged from these experiments, as Stewart, Rogoff, and Gibson (16) have pointed out that there is no necessary relation between the store of epinephrine in the suprarenals and their output; and Kuriyama (17) has also recently come to the conclusion that the secretory activity of the suprarenal medulla cannot be judged by its epinephrine content. Nevertheless, with the epinephrine content of the suprarenals remaining practically normal during sensitization and anaphylactic shock, it is highly improbable that there is any relation between these phenomena and the chromaffin substance of the suprarenals, such as has been claimed by some authors for surgical shock.

CONCLUSION.

The epinephrine store of the suprarenal glands remains unaltered in guinea pigs and rabbits which have been sensitized to ox serum, or subjected to varying degrees of anaphylactic shock by the reinjection of the foreign protein.

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EXPLANATION OF PLATE 89.

FIG. 1. Epinephrine content of the suprarenal glands determined by the biological method. *B* was a solution of adrenalin chloride, 1 cc., containing 0.01 mg. *C* contained 0.001 mg. per cc. The suprarenals of Guinea Pig 115 (Experiment 26, Table II) were extracted as for the colorimetric method, and diluted 10 and 100 times respectively.

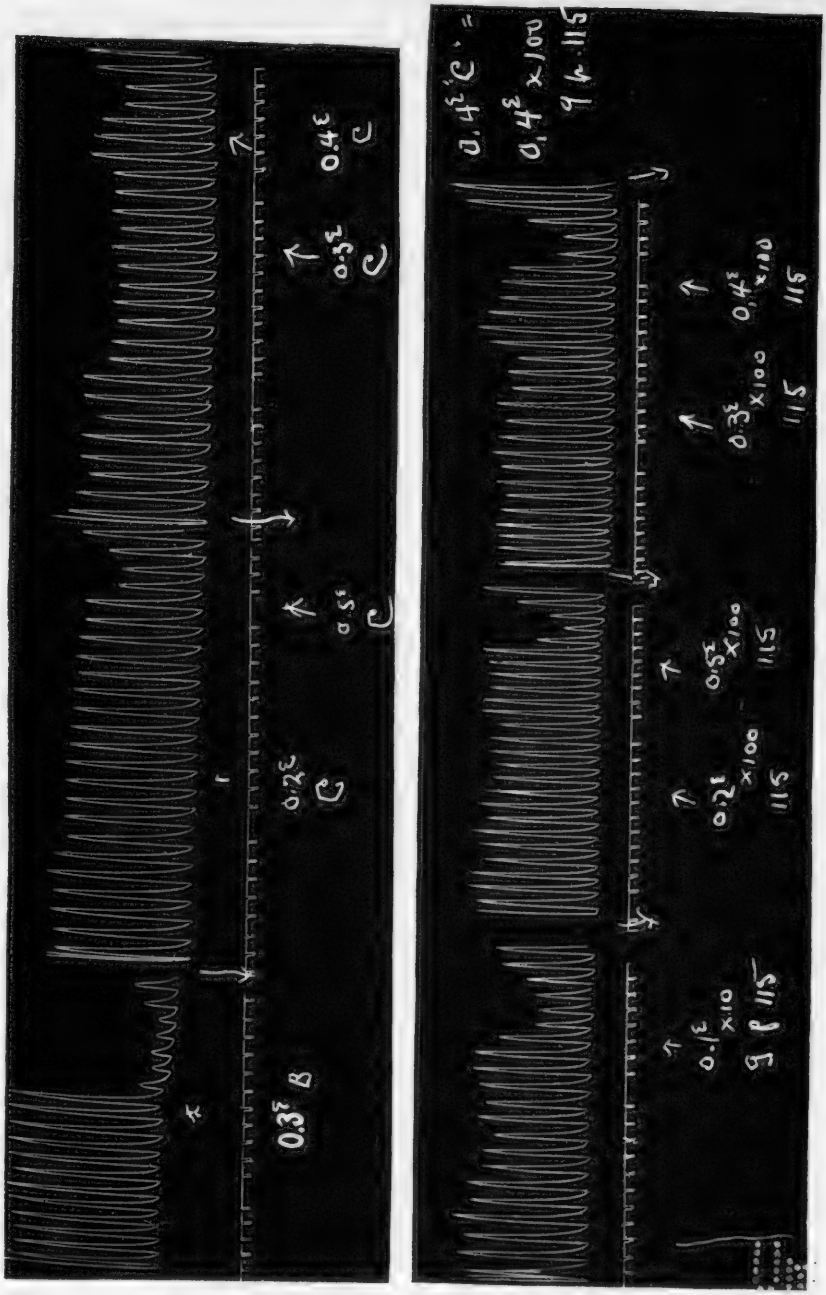

FIG. 1.

(Smith and Ravitz: Suprarenal glands in anaphylaxis.)

EXPERIMENTAL STUDIES ON YELLOW FEVER OCCURRING IN MERIDA, YUCATAN.

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INTRODUCTION.

In 1918 a leptospira was detected in the blood and liver from certain cases of yellow fever in Guayaquil.[1] The organism was demonstrated also in the blood and in organ emulsions from guinea pigs which had been inoculated with blood or liver emulsions from yellow fever patients and had shown typical symptoms of the disease, fever, hemorrhages, jaundice, and nephritis. A pure culture of the same organism was made directly from the blood of yellow fever cases and the blood of experimentally infected guinea pigs, and the pathogenicity of the culture was demonstrated in guinea pigs, marmosets, and pups. The organism, which has been designated *Leptospira icteroides*, was found to be a filter passer, easily destroyed by a temperature of 55°C. within 5 minutes, an obligatory aerobe, and requiring for growth a certain amount of unmodified blood serum from man or suitable animals. Its growth is usually suppressed by secondary bacterial contamination, but when successfully obtained it remains almost invisible to the naked eye, forming neither a discrete colony nor a striking change of the culture medium, and hence the culture may be overlooked under ordinary circumstances as sterile. An ordinary microscope does not render it visible in the fresh state, and staining with ordinary dyes fails to bring out its presence. Through a dark-field microscope with a powerful illumination, however, it can readily be recognized. In the blood of yellow fever patients the organism may be present in such small numbers that an average dark-field search usually misses it. From the comparatively small percentage of positive transmissions from yellow fever patients to guinea pigs, it seems probable that many strains of the organism fail to infect this animal fatally or even with moderate severity. Whenever a secondary bacterial infection occurs after an attempt at transmission, either the animal dies before the infection with *Leptospira icteroides* fully develops, or the course of infection is atypically modified and the strain thereby lost. The various factors just enumerated no doubt constitute some of the reasons why animal inoculation or the detection of the organism has not been successful in the hands of different investigators at an earlier period.

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxix, 547, 565, 585.

It has been shown also[2] that the majority of the serums from persons recently recovered from an attack of yellow fever possess a specific dissolving power upon the organism when tested by the Pfeiffer reaction. This phenomenon has been regarded as significant in establishing a possible relation between yellow fever and *Leptospira icteroides*. Another important point with respect to the etiological relation was furnished by the experimental transmission of the leptospira infection from yellow fever patients to normal guinea pigs, or from infected guinea pigs to normal guinea pigs, by the bite of infected stegomyia females,[3] or with an emulsion of such mosquitoes, 8 days or longer after they had fed on infected individuals or animals.

By analogy *Leptospira icteroides* presented the principal characteristics of the virus of yellow fever as experimentally determined by Reed, Carroll, Agramonte, and Lazear[4] and by later investigators (Marchoux, Salimbeni, and Simond,[5] and Parker, Beyer, and Pothier[6]). Both are filter passers, readily killed at 55°C., transmitted by *Stegomyia calopus* after a period of incubation, not amenable to cultivation in ordinary media, invisible under the ordinary microscope, not resisting bacterial putrefaction, but capable of preservation in citrated blood under a layer of liquid paraffin for several days at room temperature,[5] inoculable by hypodermic inoculation (in the case of the yellow fever virus inoculation was made into volunteer human beings,[4, 5] in the case of *Leptospira icteroides* into susceptible animals), and producing similar symptoms. From these circumstances it seemed certain that *Leptospira icteroides* was responsible for the infection in the cases from which the organism was isolated or in which a positive Pfeiffer reaction was obtained, but the question as to whether or not *Leptospira icteroides* is also responsible for the disease known as yellow fever elsewhere than in Guayaquil was left open to further experimental determination.

The present paper gives the results obtained by us during a recent expedition to Merida, Mexico.[7] Merida, a city of 100,000 inhabitants, and the capital of Yucatan, has a small proportion of non-

[2] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.

[3] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 401.

[4] Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., *Senate Doc. No. 822, 61st Cong., 3rd Sess.*, 1911, 56.

[5] Marchoux, Salimbeni, and Simond, *Ann. Inst. Pasteur*, 1903, xvii, 665.

[6] Parker, H. B., Beyer, G. E., and Pothier, O. L., *Bull. Hyg. Lab., U. S. P. H., No. 13*, 1903.

[7] This expedition was undertaken under the auspices of the International Health Board of The Rockefeller Foundation and The Rockefeller Institute for Medical Research during the months of Dec., 1919, and Jan., 1920. The Commission received hearty and efficient cooperation from the hospital and sanitary officials in Merida, to whom we wish to express our appreciation and thanks.

immune foreign population, but it owes its previous epidemics of yellow fever to the occasional influx of foreigners or of troops sent thither from other and mountainous states where there is no yellow fever. It is reported that a systematic anti-stegomyia campaign had kept the city free from yellow fever during the 4 years preceding 1919, and that it was owing to an enormous crop of stegomyias in 1919 that an epidemic broke out in July and lasted until November, claiming about 100 patients among the newly arrived troops. The death rate was about 50 per cent.

At the time when one of us arrived in Merida,[8] there had been no new case of yellow fever for 13 days. On December 15 a soldier, J. M., was brought to the yellow fever hospital in a moribund condition and died the following morning. Postmortem examination, performed 4 hours after death by Dr. A. Lara, Director of the Hospital, and Dr. Diego Hernandez, a representative of the Department of Public Health and Charities of Mexico, established the diagnosis of yellow fever. Blood from the heart and pieces of liver and kidney were obtained for experimental purposes. The details of the experiments will be given later under the heading Results obtained with Case 1.

As soon as the diagnosis of yellow fever had been established in Case 1, Dr. Hernandez ordered the soldiers (eleven in all) who had been quartered with this patient to the yellow fever hospital, there to be placed under quarantine. Among these eleven was a young soldier, A., who was apparently convalescing from a recent attack of yellow fever. The urine and serum of this individual were studied later and will be referred to in the report as Case 3.

On December 18 one of the exposed soldiers, M., began to complain of an illness which suggested strongly the beginning of yellow fever. A systematic study of this case was started at once, the blood being withdrawn from the median basilic vein at first daily and then every other day (December 18, 19, 20, 22, and 24) and used on the one hand for cultivation and on the other, if possible, for inoculation into guinea pigs. As Chart 7 shows, the fever became typical within 3 days, with the development gradually of all other symptoms, congested conjunctivæ, flushed face, muscular pains in the limbs and loins,

[8] Dr. Kligler arrived Dec. 12, 1919, Dr. Noguchi Dec. 23.

severe headache, swollen gums, nausea, epigastralgia, albuminuria, and cylindruria. On December 24 there occurred the characteristic "coffee-grounds" vomit, also definite jaundice, which became much deeper within the next several days. The albumin and casts gradually increased in the urine, and there was an abundance of bile pigment, the amount of urine being also diminished. The patient was discharged at the end of 3 weeks. Before discharge the serum and urine were collected for further study. This case will be referred to as Case 2.

RESULTS OBTAINED WITH CASE 1.

Case 1.—J. M., soldier; brought to the Casa de Salud (the yellow fever hospital) from the barracks in the night of Dec. 15, 1919, in a critical condition, with characteristic black vomit and jaundice. Death occurred at 6 a.m., and autopsy was made at 10 a.m., 4 hours post mortem.

Autopsy.—There was general jaundice, which was particularly marked in the scleras. The face was covered with black vomit. The liver was dotted with small yellowish areas. There were petechial hemorrhages in the stomach wall; the kidneys were hemorrhagic; the pericardium contained yellowish fluid; the serum was icteric.

Blood was drawn from the heart, and a portion of the liver and kidney was removed at autopsy for the experiments to be described. For the purpose of transmission guinea pigs were used throughout the present work.

Transmission Experiments with the Blood, Liver, and Kidney of Case 1.

Blood.

On December 16, 1919, intraperitoneal inoculation of 1 cc. of the heart's blood[9] was made into two guinea pigs (Nos. 1 and 2). Both had a temporary rise of temperature after 7 days but returned to normal within a few days. No jaundice developed at any period. On examination after 18 days there were some suspicious lesions in the lungs, otherwise the findings were negative.

[9] The same specimen of blood kept at 8°C. for 8 days was inoculated into Guinea Pigs 18 and 35 intracardially, but neither symptoms nor high temperature were seen to develop within 2 weeks (negative).

Kidney.

An emulsion of the kidney in sterile saline solution was injected on December 16 into two guinea pigs, one of which showed a very suggestive reaction (Guinea Pig 3).

Guinea Pig 3 (*Chart 1*).—Dec. 16, 1919. Intraperitoneal inoculation of 1 cc. of the kidney emulsion. Temperature rose to 103° F. in the afternoon of Dec. 19 and was 103° a.m. and 104.6° p.m. on Dec. 20. The animal was killed for transfers to three new guinea pigs on Dec. 21.

Transfers from Guinea Pig 3 (*Second Generation*).—December 21. 1 cc. of an emulsion prepared from the liver and kidney from Guinea Pig 3 was inoculated intraperitoneally into each of two normal guinea pigs, and 1 cc. of the heart's blood into a third. One died early of a secondary infection, and another remained well after having shown a slight elevation of temperature, while the third animal (Guinea Pig 3c) had definite signs of the leptospira infection.

Guinea Pig 3c (*Chart 2*).—Inoculation of 1 cc. of the blood of Guinea Pig 3. This animal began to show fever (103.2°) 5 days after the inoculation. The following morning the temperature remained at 103.2° and in the afternoon reached 105.6°. The animal was killed for examination and transfer.

Autopsy.—There was a light yellowish color throughout the subcutaneous tissues. The lungs showed numerous hemorrhagic foci, the gastric mucosa was highly congested, but there were no macroscopic hemorrhages in the latter. The intestines were icteric and congested, the liver was slightly icteric, the kidneys and adrenals were moderately congested, and the spleen was normal. The urine was slightly turbid and contained a considerable amount of albumin (++) and a few casts.

The blood from the heart of Guinea Pig 3c, as well as emulsions of the liver and kidney, were used to inoculate nine new guinea pigs. Two of the three inoculated with the blood and three of the six inoculated with the mixture of emulsions of the liver and kidney succumbed to a secondary infection with the paratyphoid bacillus. Two, however, of those escaping the secondary infection showed temperature curves and lesions quite typical of *Leptospira icteroides* infection, as described below.

Transfers from Guinea Pig 3c (Third Generation).—

Guinea Pig 54 (*Chart 3*).—Dec. 27, 1919. Received 1 cc. of the heart's blood from Guinea Pig 3c intraperitoneally. The temperature rose on the 7th day, and the animal remained febrile for 48 hours. It was killed for transfer and examination 10 days after the inoculation.

Autopsy.—The lungs showed numerous hemorrhagic foci. The gastrointestinal mucosa was highly congested. The liver was pale, perhaps slightly yellowish, with granular surface and several hemorrhagic spots. The kidneys were apparently swollen and degenerated, the adrenals were small and hemorrhagic, and the spleen was swollen.

Guinea Pig 59 (*Chart 4*).—Dec. 27, 1919. Received 1 cc. of a mixture of the liver and kidney emulsion from Guinea Pig 3c intraperitoneally. The temperature rose to 104° F. on the 6th day, and was 103° a.m. and 105° p.m. the following day. The animal was killed for transfer and examination on that day.

Autopsy.—There was a slight yellowness throughout the body, especially on the abdominal wall; no subcutaneous or intramuscular hemorrhages. The lungs showed several petechial hemorrhages on the posterior surface; there was a general congestion of the lobes. The liver was slightly icteric. The gastrointestinal tract showed scattered hemorrhagic areas, especially in the mucosa of the large intestine. The kidneys were highly congested, the spleen was normal, and the urine icteric.

Guinea Pigs 54 and 59 undoubtedly represented a positive transmission. The heart's blood and emulsions of the kidney and liver of Guinea Pig 59 were inoculated intraperitoneally into six normal guinea pigs, but the animals were lost through secondary bacillary infection.

Liver.

A small piece of the liver from Case 1 was emulsified in a sterile mortar with saline solution, and 1 cc. of the emulsion was inoculated into each of two guinea pigs (Nos. 5 and 6) intraperitoneally on December 16, 1919. One of them (Guinea Pig 6) presented a definite picture of the leptospira infection, while the other gave a suggestive reaction only. The protocol of Guinea Pig 6 and details regarding further transfers are given below.

Guinea Pig 6 (*Chart 5*).—Dec. 16, 1919. Received 1 cc. of the liver emulsion intraperitoneally. As the chart shows the temperature rose sharply (103° F.) in the afternoon of the 7th day and still more the next day (104° a.m. and 104.9° p.m.). On the 9th day it fell to 100.5° with the simultaneous appearance of a

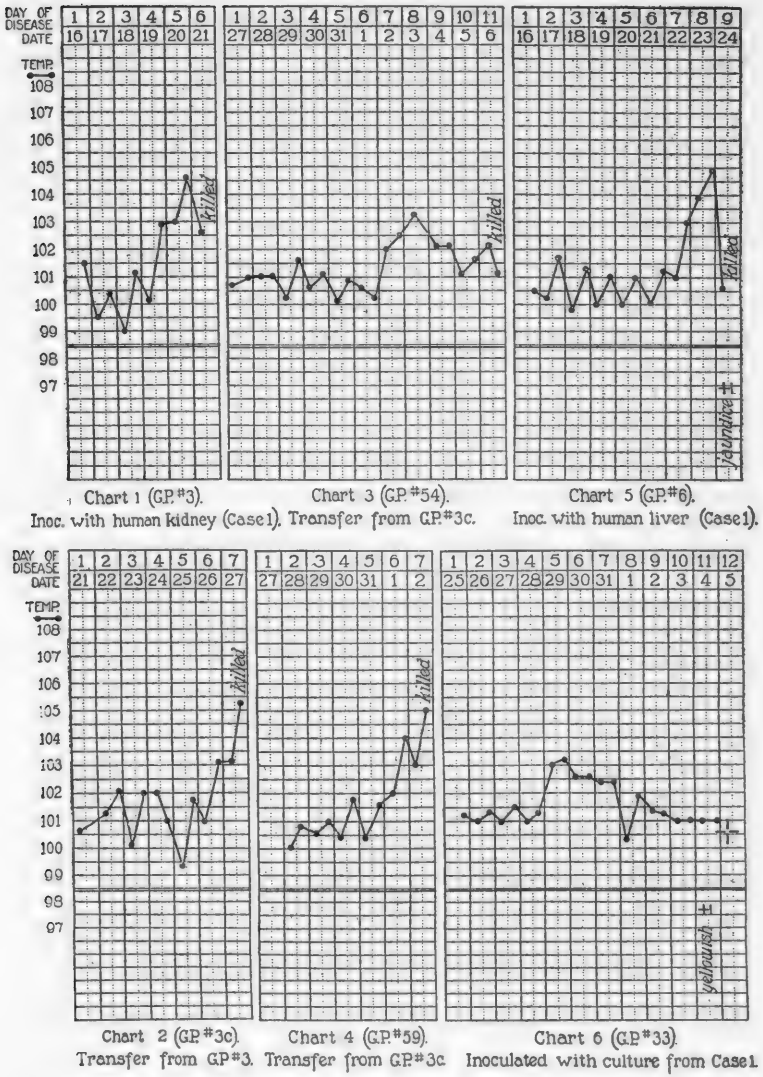


Chart 1 (G.P.#3). Inoc. with human kidney (Case1).

Chart 3 (G.P.#54). Transfer from G.P.#3c.

Chart 5 (G.P.#6). Inoc. with human liver (Case1).

Chart 2 (G.P.#3c). Transfer from G.P.#3.

Chart 4 (G.P.#59). Transfer from G.P.#3c.

Chart 6 (G.P.#33). Inoculated with culture from Case1.

CHARTS 1 to 6. Transmission experiments with autopsy material from Case 1.

trace of jaundice in the scleras and abdominal wall. The animal was killed for transfer and examination.

Autopsy.—Faint jaundice throughout the body. Lungs showed a few ecchymoses and infarctions. Liver congested, with numerous subcapsular hemorrhagic mottles, perhaps more brownish than normally. Stomach and intestines highly congested. Kidneys and adrenals highly congested, the former showing a parenchymatous degeneration. Spleen normal. Urine turbid, brownish, containing casts and albumin.

Guinea Pig 6 represented a positive transmission, and from this animal transfer was made to three normal guinea pigs (Nos. 19, 27, and 28). The results obtained in these three animals were, first, a definite febrile reaction on the 4th day, followed by an irregular fluctuation of temperature during the several successive days (indicative of a secondary infection) without jaundice at any time. These animals were killed on the 13th day for examination. The lungs showed more or less numerous hemorrhagic foci; the other organs appeared normal. No further passage was made with this material.

Transmission Experiments by Means of Cultures from the Blood of Case 1.

Attempts were made to obtain a culture of *Leptospira icteroides* with the blood obtained from Case 1. Into each of a series of culture tubes containing the medium previously found suitable[10] for the growth of *Leptospira icteroides* 0.1 to 0.5 cc. of the blood was introduced, and the tubes were placed in a thermostat, the temperature being about 26°C. A portion of blood was also mixed with sodium citrate saline solution (equal parts) and after being kept at room temperature (26°C.) for 24 hours was inoculated in amounts of 0.1 to 0.5 cc. into three tubes on December 17, 1919.

Nearly all these tubes were found to be contaminated with a *coli*-like bacillus, owing undoubtedly to a terminal or postmortem invasion by this organism of the blood circulation. On December 25 two culture tubes, inoculated on December 17, appeared to be free from any secondary bacterial contamination. Under the dark-field microscope no leptospira could be found. From each of these two tubes about 1.5 cc. of the uppermost layer of the medium, just beneath

[10] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

the paraffin oil, were taken out by means of small sterile pipettes and the mixture of the two cultures was used for inoculating three guinea pigs. Two of the animals died of a secondary infection within 2 days, and the third (Guinea Pig 33) was found dead on the 12th day after having had slight fever from the 5th to 7th day. The protocol of this animal is given below.

Guinea Pig 33 (*Chart 6*).—Dec. 25, 1919. Inoculated intraperitoneally with 1 cc. of the mixture of Culture Tubes 1 and 2 which had been kept 8 days at room temperature after inoculation with citrated blood from the heart of Case 1 at autopsy. The temperature rose to 103° F. a.m. and 103.2° p.m. on the 5th day, and was 102.6° and 102.4° respectively for the 2 following days. It returned to normal on the 10th day, and death occurred during the night of Jan. 4, 1920, 11 days from the time of inoculation.

Autopsy.—The cadaver had undergone considerable postmortem decomposition, owing to the warm temperature of the laboratory, when found lying dead in the cage on Jan. 5. Notwithstanding postmortem discoloration, there was a slight but distinct yellowness in the skin and scleras. On opening the body also the postmortem changes greatly interfered with the efforts to determine any characteristic lesions. The lungs were congested and showed a few old hemorrhagic areas. There was a small localized abscess on the abdominal wall, and the peritoneal cavity contained some turbid fluid.

It is difficult to determine whether or not the temperature curve and lesions in Guinea Pig 33 were due wholly to the abscess or in part to a mild leptospira infection.

RESULTS OBTAINED WITH CASE 2.

Case 2 (*Chart 7*).—P. M., soldier; exposed to infection through the preceding case. Dec. 17, 1919, 5 p.m. Complained of headache. Dec. 18. Increase in general malaise; headache; rachialgia; pains in the limbs. Blood was drawn at 4 p.m. Temperature 100.9°; pulse 96. No albumin in urine. Dec. 19. Temperature 101.5°; pulse 92. Blood drawn in the morning; citrated and inoculated into two guinea pigs. Trace of albumin. Dec. 20. Temperature 103.5°. Blood drawn and inoculated into three guinea pigs. Albumin 0.05 gm. Dec. 21. Trace of albumin. Dec. 22, 3 p.m. Temperature 99.3°; pulse 78. Conjunctiva injected; scleras slightly icteric. Vomited. Stool chocolate color. Urine turbid; albumin 0.18 gm. Blood drawn for culture. Dec. 23. Albumin 1.4 gm. Pulse 72. Dec. 24. Albumin 1.8 gm.; casts and renal epithelium. Jaundice marked. Blood drawn for culture and animal inoculations. Pulse 72. Dec. 25. Albumin 2.2 gm. Dec. 26. Albumin 5.5 gm. Dec. 27. Albumin 0.8 gm.

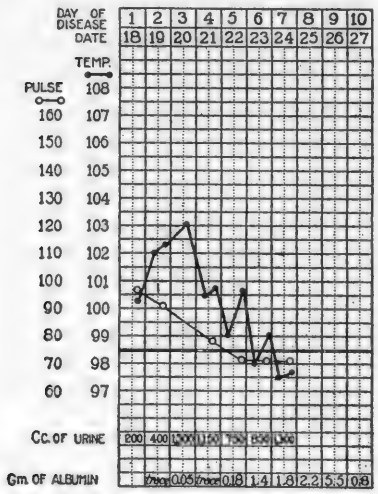


Chart 7 (patient).
Temperature curve, Case 2.

Transmission Experiments with Blood of Case 2.

First Specimen of Blood, Drawn on the 2nd Day of the Disease, December 19, 1919.

1 and 2 cc. respectively of the citrated blood were inoculated intraperitoneally into two guinea pigs (Nos. 7 and 8).

Guinea Pig 7.—The animal had a temperature of 103° F. on the 5th day; during the following 48 hours it was normal but rose again to 102.8–102.5° for 3 succeeding days. On the 12th day it was normal. On the skin of the abdominal wall were several ecchymotic areas. The animal was killed for examination.

Autopsy.—With the exception of one small hemorrhagic spot in the lungs nothing abnormal was found.

Notwithstanding this apparently negative finding, however, the blood, as well as emulsions of the liver and kidney of this animal, were inoculated into six normal guinea pigs, the blood into one and the mixed emulsions of the liver and kidney into five. The results were of doubtful nature with respect to positive transmission, although two of these animals showed some punctiform hemorrhages in the lungs when examined after 14 days.

Guinea Pig 8 (*Chart 8*).—This experiment was a duplicate of the experiment with Guinea Pig 7; that is, the animal was inoculated with the blood taken on

Dec. 19, 1919. As Chart 8 shows, the temperature rose to 104° F. in the afternoon of the 4th day, remaining high for the following 24 hours. It fell to 100.5° on the 6th day, when it seemed advisable to make a transfer.

Autopsy.—The examination showed a few hemorrhagic foci in the lungs, highly congested liver and kidneys, and a few hemorrhagic areas in the mucosa of the intestines.

Transfers with the blood and organ emulsions (liver and kidneys) of Guinea Pig 8 were made to several normal guinea pigs.

Transfers from Guinea Pig 8 (Second Generation).—

Guinea Pig 20 (Chart 9).—Dec. 24, 1919. Inoculated with the citrated blood from Guinea Pig 8, 1 cc. intraperitoneally. The temperature rose suddenly to 105° on the 5th day and remained at that point during the day. It fell to 103° the next day and to 101° on the 8th day. At this period the scleras seemed slightly icteric. The urine diminished to less than 2 cc. and contained albumin. The condition remained unchanged for the 3 succeeding days, the urine still containing albumin. Casts appeared on the 10th day. The yellowness in the conjunctiva was definite on the 11th day, and the animal died in the afternoon after subnormal temperature.

Autopsy.—Small hemorrhagic and congested areas in the lungs; liver smaller in size (?) and reddish brown with yellow mottles; kidneys unusually pale, and the demarcation between cortex and medulla indistinct. The spleen was somewhat enlarged, perhaps owing to secondary infection, and granular. In the liver and kidney stained by Levaditi's method fairly numerous leptospiras were demonstrated.

Guinea Pig 21.—Dec. 24, 1919. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions of Guinea Pig 8. Died of a secondary infection, but showed the presence of a mild leptospira infection, with albumin and casts in the urine.

Guinea Pig 22.—This experiment was a duplicate of the foregoing experiment. Temperature 103° F. on the 4th day, and fever continued for several days. Jan. 5, 1920. The animal was killed for examination.

Autopsy.—The lungs showed old hemorrhagic areas, but the other organs seemed normal.

The remainder of the citrated blood of December 19 was preserved in the ice box (about 10°C.) and after 5 days was inoculated intracardially into two normal guinea pigs, with negative results.

Second Specimen of Blood, Drawn on the 3rd Day of the Disease, December 20, 1919.

1 cc. of the citrated blood drawn on December 20 was inoculated intraperitoneally into three guinea pigs (Nos. 9, 10, and 11) on the same day.

Guinea Pigs 9 and 11.—These two animals had suggestive and almost identical temperature curves.

Autopsy.—When killed for examination on the 15th day, there were several foci of recent hemorrhage in the lungs, and the kidneys were congested; the other organs appeared normal.

Guinea Pig 10 (*Chart 10*).—This animal presented a more definite febrile reaction. On the 14th day it had a normal temperature and appeared well. It was killed for examination on that day.

Autopsy.—There were numerous hemorrhagic foci in the lungs; the abdominal wall and subcutaneous tissues were icteric and showed several large hemorrhagic areas. The adrenals were hemorrhagic, the kidneys highly congested, and the liver was apparently normal. There were pin-point hemorrhages in the gastric mucosa, and hemorrhages in the intestinal mucosa. The spleen appeared normal.

The remainder of the citrated blood of December 20 was preserved in the ice box (about 10°C.) for 4 days and 1 cc. was inoculated intracardially into each of two normal guinea pigs (Nos. 14 and 15).

Guinea Pig 14.—Sudden rise of temperature to 104° F. in the afternoon of the 4th day, but it dropped to normal (102°) on the next day. On the 6th day there was another rise to 103.5°, from which the temperature gradually fell to subnormal on the 8th day. The urine gradually diminished in quantity after the 4th day, and albumin and casts were present for several days. Ecchymotic hemorrhages were observed on the abdominal wall on the 8th day. There was a suspicion of jaundice. The animal rapidly recovered and was normal on the 13th day.

Guinea Pig 15.—Lost through secondary infection.

Third Specimen of Blood, Drawn on the 5th Day of the Disease, December 22, 1919.

The citrated blood from Case 2 drawn on December 22 was kept on ice for 48 hours (about 10°C.) before it was inoculated into two guinea pigs (Nos. 16 and 17) on December 24.

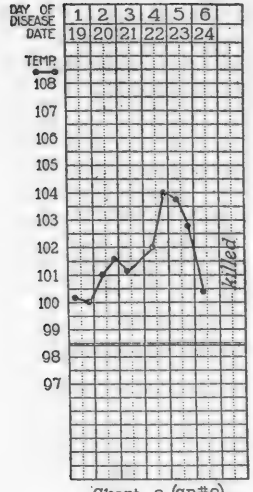


Chart 8 (G.P.#8).
Inoc. with 2nd day blood.

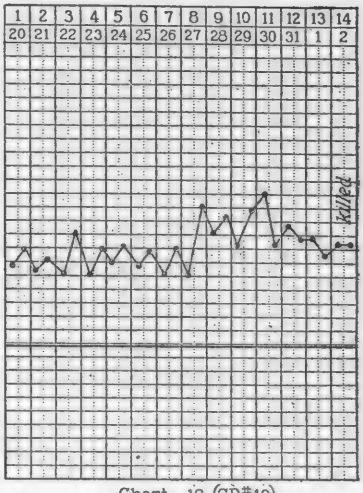


Chart 10 (G.P.#10).
Inoculated with 3rd day blood.

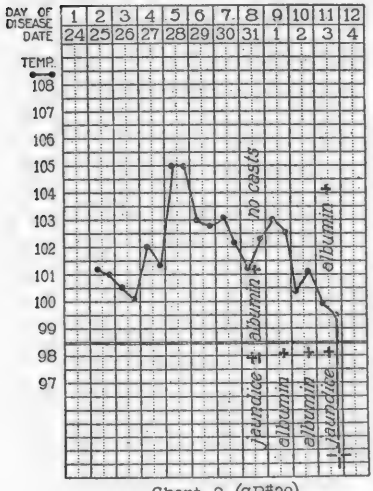


Chart 9. (G.P.#20).
Transfer from G.P. #8.

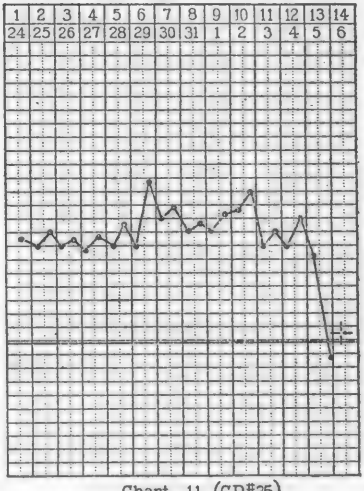


Chart 11 (G.P.#25).
Inoculated with 7th day blood.

CHARTS 8 to 11. Direct transmission experiments with blood from Case 2.

Guinea Pig 16.—Showed a rise of temperature to 103° F. on the 4th day, a decline to 102.5° on the 5th day, and a rise again to 103.5° on the 6th. At this time the animal had congested conjunctivæ. On the 9th day the temperature became somewhat subnormal but soon returned to normal during the succeeding days. The animal was killed on the 13th day.

Autopsy.—A number of hemorrhagic foci were found in the lungs, the liver appeared very pale and brownish, the kidneys were somewhat congested, the spleen normal. The animal was probably recovering from an abortive infection.

Guinea Pig 17.—This animal had practically the same febrile reaction as Guinea Pig 16.

Autopsy.—Performed on the 13th day. The organs appeared normal, with the exception of a few hemorrhagic foci in the lungs.

Fourth Specimen of Blood, Drawn on the 7th Day of the Disease, December 24, 1919.

The citrated blood was intracardially inoculated into three guinea pigs (Nos. 24, 25, and 26) within 3 hours from the time of withdrawal, each animal receiving 0.2 to 0.5 cc.

Guinea Pigs 24 and 25 (*Chart 11*).—Both animals had a suggestive temperature curve but had no jaundice at any period.

Autopsy.—The lungs showed some suspicious recent hemorrhagic areas; the kidneys were rather congested. The other organs were normal. Results inconclusive.

Guinea Pig 26.—Perhaps died of secondary infection.

Transmission Experiments by Means of Cultures from the Blood of Case 2.

Cultures were made with the citrated blood of Case 2 by introducing into a series of six tubes containing the necessary culture media[11] quantities of the blood varying from 0.1 to 0.5 cc. and adding to each a layer of paraffin oil. The tubes were kept for several days at a temperature of 26–28°C. Then, since the leptospira grows only in the presence of oxygen, the upper layer of the semisolid medium was removed from each tube by means of a sterile pipette and the contents of two or more tubes were mixed in a Petri dish. Several guinea

[11] This consisted of a rabbit serum diluted three times with 0.9 per cent sodium chloride solution and rendered semisolid by the addition of 0.1 to 0.3 per cent nutrient agar (melted). Each tube contained about 6 cc. of the mixture.

pigs were inoculated with the same material. For every series of cultures made from any given specimen of blood at least two sets of guinea pigs were inoculated, and three sets if there was a larger number of tubes.

The cultures set up with the specimens of blood taken on five occasions (December 18, 19, 20, 22, and 24) remained for the most part free from any secondary contamination during the first 5 days. Shortly afterwards, however, darkening of the hemoglobin in several tubes indicated the presence of some sort of contamination, and microscopically a fungus growth was found. Unless a surface growth of fungus was already evident, however, the contents of these tubes were also used for inoculation, though tubes showing bacterial contamination were discarded. Such contamination was rarely met with in experiments with the blood of Case 2, and that occasionally occurring was probably due to technical error. Following are the results of animal inoculations with the various culture series.

Culture, First Series. Blood Drawn on the 1st Day of the Disease, December 18, 1919.

Dark-field examination of Tubes 1 and 2, 7 days after the cultures had been set up, revealed no organism. The contents of these two tubes (2 cc. of the uppermost layer of medium from each) were mixed together in a Petri dish, and 1 cc. of the mixture was inoculated intraperitoneally into each of three normal guinea pigs (Nos. 36, 37, and 38). No definite reaction followed the inoculations in Guinea Pigs 36 and 38, which, on examination 15 days after inoculation, presented nothing remarkable in any organ except for a congestion of the lungs.

Guinea Pig 37.—Slight rise in temperature on the 7th day (102.5° F. a.m., 103° p.m.), with return to normal the next day. On the 14th day the temperature rose suddenly to 105° a.m. and 103° p.m.; on the 15th day it was 102.6° a.m. The animal was killed the same day for examination.

Autopsy.—The lungs were typical, showing numerous hemorrhagic foci, but there was no other change.

Dark-field examination of Tubes 3 and 4 of the same series demonstrated the presence of a few fungi on December 25, 7 days after

inoculation. The uppermost layers of media from the two tubes were mixed and 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 39 and 40) on that day (December 25), with negative results.

Culture, Second Series. Blood Drawn on the 2nd Day of the Disease, December 19, 1919.

Five culture tubes were available for these experiments. These were divided into two groups, Tubes 1 and 2, and Tubes 3 to 5. Dark-field examination of these tubes was made 6 days after the inoculation. Tubes 1, 2, 3, and 5 showed no organism; Tube 4 showed a few immotile leptospiras, some apparently degenerating. Occasional coarse, short filaments (fungus) were also found. The number of leptospiras was so small that a repeated, thorough search with a powerful illumination was necessary to find one. The results of animal inoculation follow.

The contents (uppermost layers) of Tubes 1 and 2 of the second culture series were taken out and mixed. 1 cc. of the mixture was inoculated intraperitoneally into two guinea pigs (Nos. 41 and 42) on December 25 and into two more on December 26. Two died of secondary infection; the others remained well.

The uppermost layer was taken out of Tubes 3, 4, and 5 of the second culture series, and 1 cc. of the mixture of these, which had been found by dark-field examination to contain a few leptospiras, was inoculated intraperitoneally into each of three guinea pigs (Nos. 43, 44, and 45) 6 days after the cultures had been made. The results of these inoculations were all positive, as the protocols show.

Guinea Pig 43 (*Chart 12*).—The temperature rose to 103° in the morning of the 5th day. During the next 36 hours it fluctuated between 102° and 102.5°. On the 7th day it rose to 105.2° a.m. and 103.5° p.m. The amount of urine was still undiminished. On the 8th and 9th days the morning temperature was 102° and the evening 104°; on the 10th it was 102.5° in the morning and 103.4° in the evening; on the 11th it fell rapidly to normal. The animal was decidedly jaundiced. The urine diminished daily after the 9th day, and only 3 cc. were secreted during the 24 hours from Jan. 3 to Jan. 4, 1920. Albumin and casts were present. The animal was killed for examination and transfer on the 11th day.

Autopsy.—Findings typical. Intense jaundice; extensive subcutaneous ecchymoses; lungs distinctly hemorrhagic; gastrointestinal mucosa congested with

hemorrhagic areas; liver yellowish; kidneys hemorrhagic and yellowish; spleen somewhat enlarged.[12] Dark-field examination revealed the presence of leptospiras in the blood as well as in emulsions of the liver and kidney.

Transfers from Guinea Pig 43.—Blood from the heart and a mixture of emulsions of the liver and kidney were intraperitoneally inoculated into six normal guinea pigs, the blood into Nos. 106 and 107, and the emulsion into Nos. 108, 109, 110, and 111. The protocols follow.

Guinea Pig 106.—Jan. 4, 1920. Inoculated with 1 cc. of the citrated blood from Guinea Pig 43. Temperature 104° F. on the 3rd day, fell to normal on the 4th, rose to 104° a.m., 104.5° p.m. on the 5th, remaining high for another 24 hours. On the 7th day it was 103.5° a.m. and 102.2° p.m. Jaundice and albuminuria were both distinct. On the 8th day died after subnormal temperature.

Autopsy.—All the characteristic lesions, together with signs of a secondary infection with the paratyphoid bacillus (enlarged spleen and fibrinous exudate in the peritoneal cavity).

Guinea Pig 107 (*Chart 13*).—This experiment was a duplicate of the foregoing experiment. As shown in Chart 13 the course of the infection was typical, the temperature rising to 105.2° a.m. and 104.6° p.m. on the 5th day. On the 7th day the animal was intensely yellow, with albumin and casts in the urine. It was killed for examination on the 7th day.

Autopsy.—Findings typical. The blood and organ emulsions showed a few leptospiras.

Guinea Pig 108.—Jan. 4, 1920. Inoculated intraperitoneally with 0.5 cc. of the mixture of the liver and kidney emulsions from Guinea Pig 43. This animal suffered from a secondary paratyphoid infection and showed a temperature of 104.6° in the afternoon of the 3rd day. This early fever endured for 3 days longer. On the 7th day it fell to 103.5°. On the 8th day it reached normal, and the animal died after subnormal temperature on the 9th day. No jaundice developed.

Autopsy.—Several hemorrhagic foci in the lungs. No leptospira was found.

Guinea Pig 109.—This animal must have been suffering from an intercurrent infection before the inoculation, as the temperature was 103.4° in the afternoon of Jan. 3, 1920, a day before the inoculation. After inoculation the temperature fluctuated between 102° a.m. and 102.5° p.m. during the 2 days that followed. On the 4th day it was normal. On the 5th it rose to 103.5° a.m. and 103.2° p.m., on the 6th it was 102.5°, on the 7th 101.5° a.m. and 96° p.m. Jaundice developed on the 6th day.

Autopsy.—Performed on the 7th day. The lesions were typical, hemorrhages in the lungs, stomach, and intestines, kidneys highly hyperemic with minute hemorrhages, liver yellowish. The spleen was enlarged, showing the effect of a

[12] Blood culture showed the presence of the paratyphoid bacillus.

secondary infection with the paratyphoid bacillus. The blood and organ emulsions contained leptospiras. A blood culture from the heart showed the presence of the paratyphoid bacillus.

Guinea Pig 110 (*Chart 14*).—Duplicate experiment. This animal escaped secondary infection with the paratyphoid bacillus and ran a typical course of the leptospira infection. The temperature rose to 103° in the afternoon of the 3rd day, was 102.2° a.m. and 104° p.m. on the 4th, 103.8° a.m. and 104.6° p.m. on the 5th. From the 6th day the temperature rapidly fell and was 98° when death occurred in the morning of the 7th day. Jaundice was definite on the 6th day.

Autopsy.—Findings typical. The leptospira was demonstrated in the organ emulsions. The animal had epistaxis at the time of death.

Guinea Pig 111 (*Chart 15*).—Duplicate experiment. This animal also escaped secondary paratyphoid infection and ran a course of fever similar to that of Guinea Pig 110, except that the highest temperature never exceeded 104° (4th day). On the 5th the temperature was still 103.8°. The examination of the blood showed no leptospiras. The animal was killed for examination and culture in the afternoon of the 5th day. The citrated blood from the heart was examined carefully for the leptospira, and one was found after long search. This blood was used for cultivation, which in 7 days was successfully accomplished.

Autopsy.—Examination of the organs showed that jaundice had not yet developed. The lungs, stomach, and intestines were dotted with hemorrhagic foci. The liver was congested, with yellowish areas. The kidneys were congested. Emulsions of the liver and kidney contained a small number of leptospiras.

Guinea Pig 44 (*Chart 16*).—This is one of the three guinea pigs inoculated with 1 cc. of the mixture of Culture Tubes 3, 4, and 5 of the second culture series on Dec. 25, 1919. This animal, in striking contrast with the foregoing ones (Guinea Pig 43 and its transfers) showed a rather slowly developing infection without much febrile reaction. The temperature remained entirely within the normal fluctuation until the 7th day, and on the 8th day the morning temperature was slightly above normal (102.6° F.), rising to 103.6° in the afternoon. On the 9th day the temperature was normal in the morning and somewhat lower in the afternoon, and the animal was distinctly yellowish. On the 10th day the temperature registered 101.3° a.m. and 101.2° p.m., perhaps somewhat below the normal, and jaundice was intense. The animal died during the night.

Autopsy.—Performed in the morning, Jan. 4, 1920. Intense jaundice. There were extensive subcutaneous hemorrhagic foci on the abdominal wall and in adjacent regions. The lungs were yellowish, with numerous hemorrhagic areas, and the heart was dilated and contained dark clot and blood. The liver was yellowish brown, mottled with congested areas, the gall bladder empty, the kidneys were swollen, hemorrhagic, and degenerated, deeply bile-stained, and the adrenals hyperemic. The stomach contents were bloody, and there were ecchymoses in the mucosa. The intestines contained bloody stools; the mucosa was congested and showed many hemorrhagic spots. The spleen was normal. The

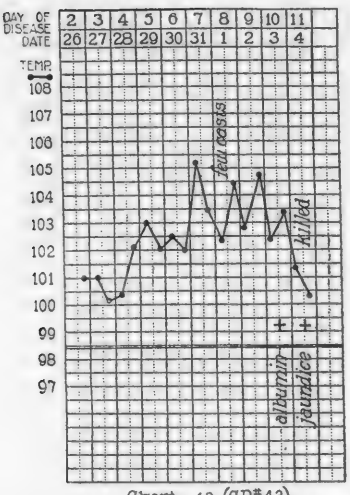


Chart 12 (G.P.#43).
Inoc. with culture of 2nd day blood.

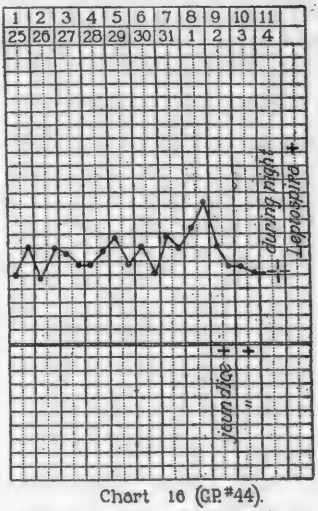


Chart 16 (G.P.#44).
Inoc. with culture of 2nd day blood.

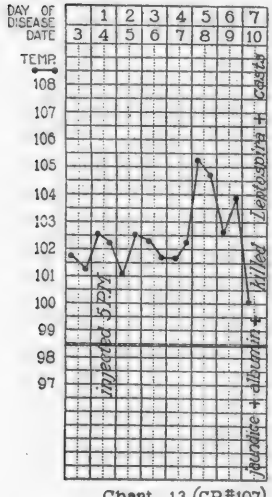


Chart 13 (G.P.#107).
Transfer from G.P.#43.

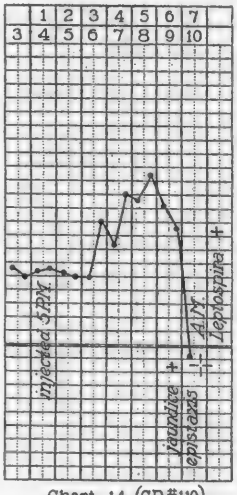


Chart 14 (G.P.#110).
Transfer from G.P.#43.

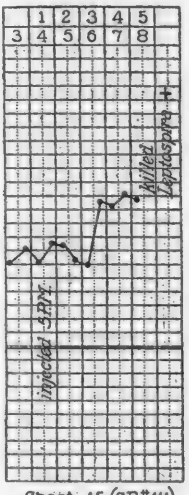


Chart 15 (G.P.#111).
Transfer from G.P.#43.

CHARTS 12 to 16. Transmission experiments with cultures from Case 2.

bladder was almost empty (anuria); ovaries and uterus congested. In the scanty urine removed from the bladder there were albumin and numerous casts with erythrocytes. The leptospira was found in the blood.

Transfer with a mixture of the liver and kidney emulsions from Guinea Pig 44 was made into Guinea Pig 105 on January 4, 1920. The result was positive in every respect. The animal succumbed to a typical leptospira infection on the 7th day, and the leptospira was detected in the blood and organs.

Guinea Pig 45.—This is the last of the three guinea pigs (Nos. 43, 44, and 45) inoculated with the same material (culture made with the blood drawn on the 2nd day) on Dec. 25, 1919. The result of the inoculation was likewise positive, although the temperature curve deviated considerably from the curves of the other two animals, owing probably to a secondary infection with another organism. On the 11th day the animal had been running a temperature of 103–104° F. for 3 days. It was killed for examination on the 11th day.

Autopsy.—The lesions were typical except for the absence of distinct jaundice. The liver was, however, somewhat yellowish. The leptospira was not found by routine dark-field examination.

Culture, Third Series. Blood Drawn on the 3rd Day of the Disease, December 20, 1919.

The citrated blood of Case 2 drawn on December 20 was introduced into five culture tubes and incubated for 5 days at 26°C. Tubes 1, 2, and 5 were free from any contamination, while Nos. 3 and 4 showed a fungus growth. A search for the leptospira by dark-field examination showed occasional immotile leptospiras in the contents from Tube 4, but was unsuccessful in the case of the other tubes.

The uppermost layer of culture medium from Tubes 1 and 2 was taken out and the two were mixed, as was done also with Tubes 3, 4, and 5. The two mixtures were inoculated into corresponding groups of guinea pigs.

Guinea Pigs 46 and 47.—Inoculated with 1 cc. of the mixture of Culture Tubes 1 and 2. Both animals had a suggestive temperature curve. On the 13th day both guinea pigs were killed for examination.

Autopsy.—Findings similar, both animals having old hemorrhagic foci in the lungs, congested stomach and kidneys, and normal spleen. The liver was slightly yellowish in Guinea Pig 46.

There was perhaps an abortive infection in both these animals.

Three guinea pigs (Nos. 48, 49, and 50) were inoculated each with 1 cc. of the mixture of Culture Tubes 3, 4, and 5. The results were highly suggestive of an abortive leptospira infection with Guinea Pigs 48 and 49, and quite definite with Guinea Pig 50, in which jaundice was also present. From Guinea Pig 48 a positive transfer to another guinea pig was also accomplished.

Guinea Pig 48.—Typical temperature rise on the 5th day, reaching 103.8° F. a.m. and 103.4° p.m. The temperature remained normal on the 6th day and continued so until the 9th day, when the animal was killed for examination and transfer.

Autopsy.—The lesions found were small, discrete hemorrhagic foci in the lungs, swelling and congestion of the kidneys, and congestion of the stomach mucosa. The other organs appeared to be normal.

Transfers were made with the citrated blood from the heart of Guinea Pig 48 into two guinea pigs and with a mixture of the liver and kidney emulsions into four guinea pigs. One of the animals inoculated with the blood showed a temperature of 102.8°F. in the afternoon of the 8th day and 103° in the morning of the 9th day, but soon returned to normal. When it was killed for examination on the 12th day there were a few hemorrhagic patches in the lungs, the liver was somewhat yellowish, and the kidneys were congested.

Transfer with the organ emulsions from Guinea Pig 48 gave a more decided picture of the leptospira infection in some but no reaction in others.

Guinea Pig 98.—Temperature of 102.8° on the 5th day, 102.5° a.m. and 103.2° p.m. on the 6th, 103° a.m. and 102° p.m. on the 7th. On the 9th the temperature became normal. The animal was killed on the 12th day for examination.

Autopsy.—The lungs showed many recent hemorrhagic patches, the mucosa of the stomach several old hemorrhagic foci. The kidneys were pale; the spleen was normal.

Culture, Fourth Series. Blood Drawn on the 5th Day of the Disease, December 22, 1919.

Only four tubes were made with the citrated blood drawn on December 22 from Case 2, and after 9 days only two tubes appeared to be free from bacterial contamination. These tubes also contained a slight fungus growth which did not change the appearance of the

medium to any marked extent. Under the dark-field microscope two undoubted immotile leptospiras were found in the mixture of the contents of the two tubes. 1 cc. of the mixture was also inoculated into each of four guinea pigs.

The results of this series of inoculations were unsatisfactory, owing to a secondary infection in all the guinea pigs.

Culture, Fifth Series. Blood Drawn on the 7th Day of the Disease, December 24, 1919.

Six culture tubes were inoculated with the citrated blood of this date. Unfortunately the tubes were covered with an accidentally contaminated paraffin oil, and within a few days incubation at 28°C. all of them contained a profuse growth of fungus.

SUMMARY.

Injections into guinea pigs of the blood and the emulsions of liver and kidney obtained at autopsy from a fatal case of yellow fever in Merida induced in some of these animals, after a period of several days incubation, a rise of temperature which lasted 1, 2, or more days. When killed for examination at this febrile stage the animals invariably showed hemorrhagic areas of various size, sometimes few and sometimes numerous, in the lungs, and also, though less constantly, in the gastrointestinal mucosa, together with general hyperemia of the liver and kidneys. In a guinea pig (No. 6) inoculated with the liver emulsion of Case 1 there was a trace of jaundice on the 9th day. Injections of the blood or liver and kidney emulsions from such animals into normal guinea pigs reproduced the febrile reactions and the visceral lesions. The majority of the animals which were allowed to live and complete the course of the infection rapidly returned to normal (within several days). Examinations of these surviving guinea pigs after 2 weeks revealed the presence of rather old hemorrhagic foci in the lungs.

In the course of further attempts to transfer the passage strain, a secondary infection by a bacillus of the paratyphoid group caused many deaths among the guinea pigs and resulted finally in the loss of the strain from Case 1.

Most of the cultures made with the heart's blood taken at autopsy from Case 1 proved to be contaminated with a bacillus of the *coli* group. The contents of the apparently uncontaminated tubes were inoculated into guinea pigs, but the results were for the most part negative or vitiated by a secondary infection.

Dark-field search for the leptospira with the autopsy materials was negative, although prolonged and thorough examination was not practicable at the time of these experiments. Our efforts were concentrated on obtaining positive animal transmission rather than on the time-consuming demonstration of the leptospira, which when unsuccessful does not necessarily exclude the presence of the organism in small numbers. Likewise, the dark-field work with the material from guinea pigs was confined to a brief examination and was omitted in many instances. Under these circumstances no leptospira was encountered in any of the material from Case 1.

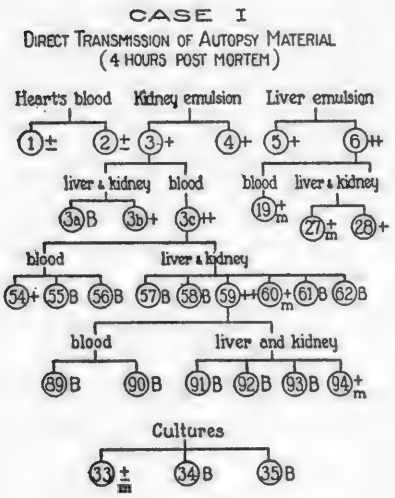
On the other hand, the results obtained with the specimens of blood from Case 2 were definitely positive, not only in the transmission of the disease directly, or indirectly by means of cultures, into guinea pigs, but also in the demonstration of the leptospira in the primary cultures and in the blood and organ emulsions of guinea pigs experimentally infected with such cultures.

Definite positive direct transmissions were obtained with the specimens of blood drawn on the 2nd and 3rd days. No blood was taken on the 4th or 6th days. There were indications of abortive or mild leptospira infection in the guinea pigs inoculated with the blood taken on the 5th day.

Regarding the inoculation of cultures from Case 2, it may be stated that only the cultures (leptospira +) made with the blood drawn on the 2nd day caused a definite fatal infection in guinea pigs. From this series a continuous passage in the guinea pig has been successfully accomplished. One of the guinea pigs (No. 48) inoculated with the culture 5 days old (leptospira +) made from the blood taken on the 3rd day presented typical symptoms, and a positive transfer from this to another animal (No. 98) was also made. Cultures of the blood drawn on the 5th and 7th days gave unsatisfactory results, owing to a secondary contamination. Leptospiras were detected in some of the culture tubes containing 2nd and 3rd day

specimens of blood from Case 2; they were few in number and for the most part immotile, owing perhaps to some unfavorable cultural condition such as a fungus contamination.

Charts 17, 18, and 19 give a summary of the experiments.



\+ Temperature & lesions typical.
++ All symptoms, including jaundice & lesions.
+m Mixed infection.
± Doubtful leptospira infection.
− Negative result in regard to leptospira infection.
++m Severe leptospira infection with all symptoms, but concomitant secondary infection.
B Bacterial infection.
L. Leptospira.

Chart 17.

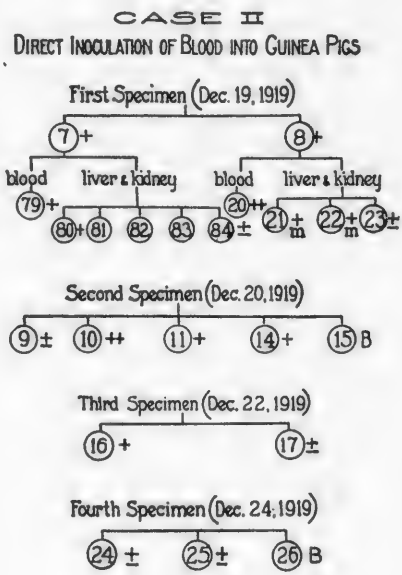


Chart 18.

CASE II

TRANSMISSION BY MEANS OF CULTURES FROM BLOOD

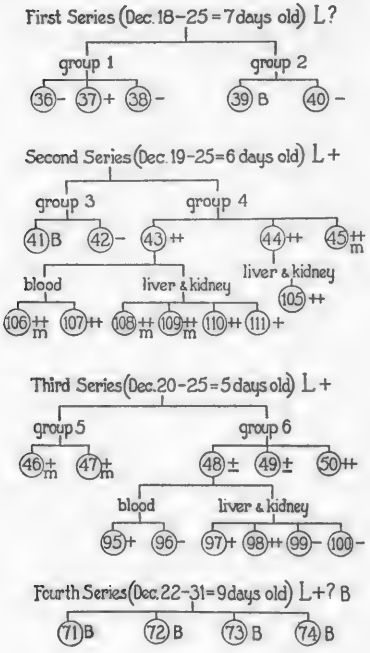


Chart 19.

IMMUNOLOGICAL STUDIES WITH A STRAIN OF LEPTOSPIRA ISOLATED FROM A CASE OF YELLOW FEVER IN MERIDA, YUCATAN.

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Morphology, Cultural Properties, and Virulence of the Strain of Leptospira Isolated in Merida.

The morphological features and cultural properties of the strain of *Leptospira icteroides* isolated in Merida are similar to those of the Guayaquil strains.[1] With respect to pathogenicity and virulence we obtained the following data.

Guinea Pig 112 was inoculated on January 4, 1920, with the mixed emulsions of liver and kidney from Guinea Pig 43, which had typical symptoms as a result of inoculation of a culture made with blood drawn from Case 2 on December 19, 1919. No. 112 came down with typical symptoms on the 8th day and was killed for transfer on January 12. The liver and kidney were emulsified together with 0.9 per cent saline solution, about 1 gm. of organ material to 10 cc. of saline solution. The emulsion was allowed to stand several minutes until the supernatant fluid was free from coarse tissue particles. The clear portion, which under the dark-field microscope showed a few leptospiras in every field, was used to determine the minimum lethal dose for guinea pigs. The procedure consisted in inoculating intraperitoneally graduated amounts of the emulsion into as many guinea pigs as the number of dilutions required, each amount being contained in a uniform volume of 1 cc. of 0.9 per cent saline solution. The results of the experiment are recorded in Table I.

The period of incubation varied from 3 to $7\frac{1}{2}$ days, and death occurred 7 to 10 days after the time of inoculation. The duration

[1] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 13.

of illness, calculated from the beginning of fever until death, varied from $2\frac{1}{2}$ to $4\frac{1}{2}$ days. We did not succeed in finding the minimum lethal dose, which apparently lies below 0.0001 cc., the smallest quantity of emulsion employed in the present experiment.

The Merida strain showed a marked pathogenicity for young pups, 6 weeks old, when given intraperitoneally.[2]

TABLE I.

Determination of the Minimum Lethal Dose of the Merida Strain.

Guinea pig No.	Amount of organ emulsion.	Length of incubation.	Time of death.
	cc.	*days*	*days*
163	0.5	3	7
164	0.2	$3\frac{1}{2}$	8
165	0.1	$3\frac{1}{2}$	7
166	0.01	5	8
167	0.001	$7\frac{1}{2}$	10
168	0.0001	5	9

Dog 1.—Jan. 14, 1920. Inoculated with 4 cc. of the kidney emulsion from Guinea Pig 102 (second passage in guinea pigs) intraperitoneally. The temperature was 39.6°C. on the 4th day, falling rapidly to 38° the next morning. In the afternoon of the 7th day the animal was found near collapse (37°) and was killed for examination. Jaundice had been noticed in the conjunctivæ on the 6th day, and there was general jaundice on the 7th day. Albuminuria began on the 5th day and increased until the animal was killed.

Autopsy.—General icterus; marked congestion of the lungs and other organs. Hemorrhagic foci were found in small number in the lungs and intestinal mucosa. The kidneys were congested and degenerated. The liver was mottled with yellowish patches. No subcutaneous or intramuscular hemorrhages were found. The serous membranes were free from hemorrhage.

Dog 2.—This animal received the same material as Dog 1 at the same time. The temperature curve was somewhat more irregular, 39°C. on the 2nd and 3rd days, normal on the 4th day, and 39.6° on the 5th day. The decline was very rapid, 37.5° a.m. and 36.2° p.m. on the 6th day. The animal died during the night. Albuminuria was present on the 4th day. Jaundice was noticeable on the 5th day and had definitely increased during the 6th day.

Autopsy.—General jaundice; liver and other organs yellowish. Few small hemorrhagic foci in the lungs. The stomach contained some digested blood,

[2] Villamil Mendoza, M., Apuntes acerca de la fiebre amarilla, Thesis, Merida, 1920.

and the mucosa of the intestines was profusely hemorrhagic. The kidneys were congested and degenerated. Subcutaneous tissues, muscles, and serosa not hemorrhagic.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

The identity of the Merida strain of the leptospira was established by means of a series of experiments in which varying quantities of anti-*icteroides* serum were injected, together with 1 cc. of an emulsion of the liver and kidney from Guinea Pig 43, into several normal

TABLE II.

Identification of the Merida Strain and the Therapeutic Value of Anti-icteroides Serum against This Strain.

Guinea pig No.	Amount of serum.	Result.	Lesions.
	cc.		
109	0 (control).	Killed after 6 days.	Typical.
110	0 (").	Died " 6 "	"
111	0 (").	Killed " 4 "	" lung lesions.
112	0.1	Survived.	
113	0.01	"	
114	0.001	"	
115	0.0001	"	

guinea pigs. The anti-*icteroides* serum had been prepared in a horse by repeated injections of different strains of *Leptospira icteroides* from Guayaquil cases. The results are recorded in Table II.

The outcome of this experiment made it evident that the Merida strain belongs to the same group as the Guayaquil strain.

Polyvalent anti-*icteroides* immune serum prepared in the horse or monovalent anti-*icteroides* immune serum prepared in the rabbit had a definite devitalizing action upon the Merida strain, while antiserums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serotherapeutic Experiments.

The purpose of the next series of experiments was to ascertain whether or not the same immune serum possessed a therapeutic value in infection with the Merida strain such as had been demonstrated in the case of experimental infection in guinea pigs with the Guayaquil strains.[3] Thirty-one guinea pigs were inoculated at the same time with 0.5 cc. of the organ emulsion, representing at least 5,000 minimum lethal doses. 1 hour after inoculation two of the animals were given an injection of 0.0001 cc. of the serum, and two more received an injection of 0.1 cc. The procedure was continued with each group of four animals after periods of 24, 48, and 72 hours and 4 and 5 days respectively. After 5 days the amounts of serum injected were increased to 1 and 2 cc., as it had been found that the smaller doses had no effect at this period of the disease.

All the animals which received no serum until after 72 hours showed fever at that time; in those untreated until 4 days there were fever and slight jaundice; those not treated until 5 days showed a decline in temperature and increasing jaundice; and in the group which received the injection after 6 days there were intense jaundice and subnormal temperature, and the animals were near or in collapse. The results of this series are summarized in Table III.

0.1 cc., therefore, of the anti-*icteroides* serum prevented any external manifestation of the infection if given before the onset of fever (within 72 hours after inoculation). The same dose, given to animals in the febrile stage, but without jaundice, prevented the development of jaundice; animals still having fever and showing more or less jaundice at the time of injection of this dose likewise survived, and in some jaundice rapidly disappeared in 24 hours. On the other hand, this quantity of serum failed to prevent a fatal outcome when given to animals in which defebrescence and increasing jaundice had set in. The nephritic symptoms, which had existed since 72 hours after inoculation, were rapidly increasing by this time.

0.0001 cc. of the serum prevented a fatal infection in three out of four guinea pigs when given within 24 hours. In one animal which

[3] Noguchi, H., *J. Exp. Med.*, 1920, xxxi, 159.

died from the typical infection there was a prolongation of the incubation period (6 days) and the duration of illness (5½ days). In two of the three surviving animals fever developed after 4 days, but they recovered without showing jaundice at any time. Injection of this quantity of serum 48 hours after inoculation, or later, had no effect upon the course of the disease, all animals so treated dying within 5 to 8½ days with typical symptoms.

Seven guinea pigs which were near or in a state of collapse 6 days after the inoculation were given 1 cc. (three animals) and 2 cc. (four animals), but with one exception all died within ½ to 2½ days from the time of injection of the serum.

It may therefore be concluded that the anti-*icteroides* serum here employed is able, in a dose of 0.1 cc., to protect a guinea pig from a fatal infection against at least 5,000 minimum lethal doses of the Merida strain when administered during the incubation period or at any early stage of the disease (fever and beginning of jaundice), but that it has almost no effect upon the course of the disease if given at a later period when the animals are deeply yellow and the temperature has begun to go down. At or near collapse even 1 or 2 cc. failed to prevent death in the majority of animals. On the other hand, a quantity as minute as 0.0001 cc., when given within 24 hours, is able to protect the majority of animals from a fatal infection.

If man's degree of susceptibility to *Leptospira icteroides* is comparable to that of the guinea pig, it may reasonably be assumed that the injection of the anti-*icteroides* serum at an early period of the disease will have a beneficial effect similar to that observed in the treatment of the experimental infection in guinea pigs. On the basis of body weight a man of 80 kilos would require about 200 times the amount of serum needed to save a guinea pig of 400 gm.; that is, 20 cc. (0.1 cc. × 200 = 20 cc.). The mode of administration should be intravenous, and if necessary the injection should be repeated at short intervals (4 hours).

TABLE III.

Serotherapeutic Value of the Anti-icteroides Serum against 0.5 Cc. (5,000 Minimum Lethal Doses) of an Organ Emulsion, Merida Strain.

Length of time before serum injection.	Symptoms.	Amount of serum injected.			
		0.0001 cc.	0.1 cc.	1 cc.	2 cc.
hrs.					
1		No. 131. Incubation period 6 days. Died after 11½ days.	No. 133. No infection.		
		No. 132. No infection.	" 134. " "		
24		" 135. Fever from 6th day on, but recovered.	" 137. " "		
		No. 136. Fever on 5th day, but recovered.	" 138. " "		
48		No. 139. Died after 6½ days.	" 141. " "		
		No. 140. Died after 5 days.	" 142. " "		
72	Fever; no jaundice.	No. 143. Died after 7½ days.	" 145. Survived without jaundice.		
		No. 144. Fever on 5th day. Died after 7½ days.	No. 146. Survived without jaundice.		

days					
4	Fever; jaundice.	No. 147. Died after 8½ days. No. 148. Died after 6 days.	No. 149. Survived, but jaundice increased next day. No. 150. Survived; jaundice disappeared next day.		
5	Temperature down; jaundice.	No. 151. Died after 6 days. No. 152. Died after 5½ days.	No. 153. Died after 8½ days. No. 154. Died after 7½ days.		
6	Near or in collapse.			No. 159. Died after 7½ days. No. 160. Died after 8½ days. No. 161. Died after 8 days.	No. 155. Died after 8½ days. No. 156. Died after 6½ days. No. 157. Died after 6½ days. No. 158. Recovered.

Pfeiffer Phenomenon with the Serums of Yellow Fever Convalescents in Merida.

Pfeiffer's phenomenon with *Leptospira icteroides* had been previously observed with a limited number of serums from yellow fever convalescents in Guayaquil[4] and was positive in about 85 per cent of the cases studied. It was desirable that this line of observation should be extended to yellow fever cases existing elsewhere, and we availed ourselves of the present opportunity with the Merida cases. Our first intention was to study as many specimens of serum from yellow fever convalescents in Merida as could be obtained, with as many strains of cultures of *icteroides* as possible. This plan could not be carried out, however, for two reasons; there were only a few convalescents accessible, and all the Guayaquil cultures brought down by us to Merida were lost within a few days after our arrival, and before our work had been started, by an accidental rise of temperature for 3 days to 56°C. of the thermostat in which the cultures had been placed for safety. Hence work on the Pfeiffer phenomenon was possible only after the isolation of *Leptospira icteroides* from Case 2.

There were two convalescents at the yellow fever hospital (Casa de Salud), Cases 2 and 3.[5] Both patients (soldiers) were infected in the same house in Merida, Case 3 preceding Case 2 by perhaps 2 weeks. The serum for the Pfeiffer test was drawn at the end of the 5th week of the disease in Case 3 and during the 3rd week in Case 2. Both specimens were still distinctly jaundiced. Through the courtesy of Dr. A. Lara we were given specimens of serum from two other convalescents who had had a typical attack of yellow fever in August, 1919; that is, about 5 to 6 months previously. One was a young woman, whose urine still contained albumin at that time, but who showed no bile pigment either in the urine or in the serum.

The following procedure was employed. 0.5 cc. of each specimen of serum was mixed with 1.5 cc. of a pure culture of the Merida strain of the leptospira and the whole injected into the peritoneal cavity of a guinea pig. The exudate was withdrawn from the peritoneal cavity after 30 minutes for dark-field examination. Table IV is a record of the results.

[4] Noguchi, H., *J. Exp. Med.*, 1919, xxx, 9.
[5] Noguchi, H., *J. Exp. Med.*, 1920, xxxii, 601.

The serums from yellow fever convalescents in Merida gave uniformly a positive Pfeiffer reaction when tested with a culture of the leptospira isolated from Case 2. Although the guinea pig inoculated with the mixture of this culture and the serum from the same patient died of secondary infection too soon to permit any observation with regard to the power of the serum to protect the animal from a fatal infection, the animal which received the serum from Case 3 with this culture escaped the infection altogether. It may be supposed that this patient had been attacked by the same strain that later infected the other two soldiers (Case 1, fatal, and Case 2) living in the same

TABLE IV.

Pfeiffer Reaction with Serum from Convalescents and the Merida Strain.

Serum No.	Type of infection.	Length of time after onset of disease.	Pfeiffer reaction.	Remarks.
		wks.		
1 (Case 2).	Moderately severe.	3	Positive.	Died of intercurrent infection within 24 hrs.
2 (" 3).	Moderately severe.	5	"	No infection resulted.
		mos.		
3 (civilian).	Typical.	5	"	Died after 7 days with typical infection.
4 (").	"	6	"	Died after 7 days with typical infection.
No serum (control).			Negative.	Died after 9 days with typical infection.

house, hence such complete protection. On the other hand, Serums 3 and 4 were from two civilians who had recovered from yellow fever at least 5 months previous to the time when their blood was tested. These serums gave a positive Pfeiffer phenomenon but failed to protect the animals.

These four serums were brought back to The Rockefeller Institute to be tested with the Guayaquil strains of *Leptospira icteroides* and also with some of the strains of *Leptospira icterohæmorrhagiæ*. The serums from Cases 2 and 3 were accidentally lost during transportation. Moreover, during the journey the remaining two had to be

kept at a comparatively high temperature (heated steamer cabin and trains) for 15 days, and their activity must have suffered considerably. Nevertheless, tests were made on March 5; that is, 2 months from the time of collection. The amount available of Serum 3 was 0.75 cc. and that of Serum 4, 0.9 cc. Each specimen was divided into three equal parts and tested with two Guayaquil *icteroides* strains (Nos. 1 and 5) and with the American No. 1 strain of *icterohæmorrhagiæ*. The results obtained are presented in Table V.

TABLE V.

Pfeiffer Reaction with Serum from Convalescents Tested with Two Guayaquil Strains of Leptospira icteroides and One Strain of Leptospira icterohæmorrhagiæ.

Serum No.	*Leptospira icteroides.*		*Leptospira icterohæmorrhagiæ.*
	Guayaquil Strain 1.	Guayaquil Strain 5.	American Strain 1.
3	Positive.	Doubtful.	Negative.
4	Partial reaction.	Partial reaction.	"
No serum (control).	Negative.	Negative.	"

While no definite conclusions can be drawn from the results obtained with these old serums, it is possible to recognize an unmistakable specific reaction with the *icteroides* strains, particularly between Serum 3 and No. 1 of the Guayaquil *icteroides* strains. With Serum 4 there was not complete destruction of the *icteroides* organisms; but about one-half of them became paralyzed, and some were degenerated, although many active organisms were simultaneously present. There was no effect whatever upon American Strain 1 of *Leptospira icterohæmorrhagiæ*.

SUMMARY.

Identification of the leptospira isolated from a case of yellow fever in Merida was accomplished by means of an anti-*icteroides* immune serum prepared in a horse with several Guayaquil strains of *Leptospira icteroides*. The immune serum showed a protective action of high titer against the Merida strain, thus establishing its efficacy as a therapeutic agent against this strain. Polyvalent anti-*icteroides*

immune serum prepared in the horse or monovalent anti-*icteroides* immune serums prepared in the rabbit had a definite devitalizing action upon the Merida strain, while immune serums similarly prepared with strains of *icterohæmorrhagiæ* had no perceptible effect upon the Merida strain.

Serums from yellow fever convalescents in Merida gave a positive Pfeiffer reaction with the Merida strain of *Leptospira icteroides*. The reactions between the Guayaquil strains (Nos. 1 and 5) and two of these serums from convalescents varied from definitely positive to doubtful, owing probably to the diminution of active immune principles in the serums during the prolonged and unfavorable conditions of their transportation.